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FOREWORD

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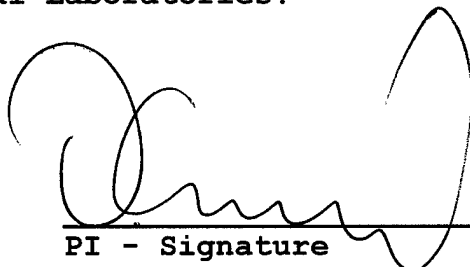
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(5). INTRODUCTION

The major goal of this proposal is the development of a conditionally replicative adenovirus (CRAD) agent for application in the context of carcinoma of the prostate. The therapeutic utility of such CRADs is based upon their specific replication within target tumor cells with oncolysis achieved on that basis. To achieve such a therapeutic end, a CRAD agent must accomplish three key steps: 1) efficient adenovirus infection of tumor cells; 2) preferential replication of the adenovirus within the tumor cells; and 3) effective evasion of the immune system. The infectivity aspect is especially critical as this parameter determines not only the effective initial inoculum achievable by intratumoral adenoviral injection, but also overall CRAD efficacy, based on the ability to achieve lateral infection for a multiplicative effect. Our initial proposal presumed that adenoviral infectivities were of sufficient efficiency for exploitation in the context of developing a prostate-specific CRAD. Indeed, adenoviruses have been employed for a variety of gene therapy strategies for carcinoma of the prostate. On the other hand, human trial data suggested that vector-mediated infection frequency, in the stringent human context, was suboptimal. Furthermore, the consequences of tumor cell resistance to adenoviral infection were recognized in the specific context of employing CRAD agents for anti-tumor therapy. It was thus obvious that until we addressed the issue of adenoviral infectivity for target tumor cells, our proposed attempts to improve CRAD specificity would not be of direct utility. On this basis, we sought to initially improve CRAD efficacy via infectivity-enhancement of the adenovirus vector. Subsequent to the achievement of this goal, we could then more logically proceed to improving the specificity of adenoviral replication. The combination of these two endeavors, infectivity enhancement and replicative specificity, would thus allow the derivation of the optimal CRAD agent for carcinoma of the prostate.

(6). BODY

A. Background

Conditionally-replicative adenoviruses (CRADs) represent a novel and promising approach to treat neoplastic diseases (1). This approach offers several conceptual advantages over conventional gene therapy approaches. First, the replicative property of these agents circumvents the need to achieve quantitative tumor cell transduction. Thus, the intrinsic amplification offered by replicative viruses potentially allows oncolysis with extensive tumor transduction. Secondly, the property of specific replication offers the potential to achieve an improved therapeutic index. On this basis, CRAD-based strategies have been rapidly translated into clinical trials.

Realization of the full utility of CRADs is subservient to their capacity to function as infectious agents in human tumors. After injection, the agent must replicate specifically, propagate laterally, and avoid the host immune response. Strategies to address each of these steps may improve the utility of these agents. To this end, a variety of approaches have been implemented to achieve tumor-specific replication (1). One approach is based on the control of the expression of an essential early viral gene using tumor-specific promoters (2,3). Another approach is based on deletions made in viral genes encoding proteins that interact with cellular proteins to complete the lytic life cycle (4).

In addition, limitations in the infectivity of tumor cells by adenoviruses can potentially curtail the initial infection and lateral propagation of CRADs. In this regard, human clinical trials with adenovirus have demonstrated relatively inefficient infectivity *in vivo*. This has been related to the paucity of the primary adenovirus receptor, coxsackie-adenovirus receptor (CAR), on tumor cells. Based on this recognition, it has been proposed that gene delivery via CAR-independent pathways may be required to circumvent this aspect of tumor biology (5,6).

To address the issue of limited infectivity of tumors by adenoviruses, we have developed methods to enhance infectivity via CAR-independent entry pathways. One approach is based on the genetic modification of the HI loop of the virus fiber (7). A virus containing a fiber modified with an RGD peptide in the HI loop has an expanded tropism because it binds the cells through RGD/integrin interactions in addition to CAR (7). Here, we show that the incorporation of the RGD motif into the fiber of a CRAD enhances its oncolytic potency. Tropism modifications such the introduction of RGD presented here, are thus clearly necessary to overcome limitations in tumor infectivity and to realize the full potential of CRADs.

B. Results

Enhancement of virus infectivity mediated by the RGD peptide incorporated into the HI loop to the fiber knob. Incorporation of RGD into adenoviral fiber has been

shown to increase adenoviral vector infectivity in a variety of cell lines and primary tumors (7,12). We have chosen three cell lines to test this concept: A549 lung adenocarcinoma, LNCaP prostate carcinoma, and SKOV3.ip1 ovarian carcinoma. A four-fold increase in transduction mediated by RGD in SKOV3.ip1 has been reported previously (13). The increase in transduction resulting from the modification of the fiber with an RGD sequence in A549 cells and LNCaP cells was determined by infection with E1-substituted vectors expressing luciferase containing the wild type fiber or the RGD-modified fiber. In both cell lines the RGD modified vector showed an infectivity advantage over the non-modified counterpart (Figure 1).

The major differences were observed in A549 cells with a 100x increase in transduction, followed by LNCaP with a 10x increase. In LNCaP the major differences were observed at lower multiplicities of infection indicating that the CAR mediated pathway was quickly saturated.

Propagation advantage of an RGD modified conditionally replicative adenovirus.

To take advantage of the increased infectivity conferred by the RGD modification in a replication-competent context, we chose an adenovirus with a deletion in the retinoblastoma-binding site of E1A. The characterization of this virus in terms of tumor-specific replication will be presented elsewhere (10). The D24 deletion of E1A, and the RGD insertion in the fiber, were combined into a unique viral genome by homologous recombination in bacteria. The resulting virus, Ad5-D24RGD, was propagated efficiently in a variety of cell lines. The presence of the D24 mutation in E1A and the RGD insertion in the fiber was analyzed by PCR (Figure 2).

Of note, we have not observed the outcome of adenoviruses with wild-type E1 or wild-type fiber throughout the propagation of Ad5-D24RGD concordant with the lack of endogenous adenoviral sequences in A549, cells that we regularly used to propagate this virus.

After structural confirmation, Ad5-D24RGD, Ad5D24, Ad5-wt, and Ad5lucRGD were compared in their ability to replicate in the three cell lines studied. Cell monolayers were infected with a small amount of each virus (0.01 m.o.i.) in the presence of ³H-thymidine. During an 8-day incubation period, the encapsidated viral DNA was isolated at days 2,4, and 8. Viral DNA corresponding to one million cells was analyzed by gel electrophoresis and quantified by scintillation counting. Ad5-D24RGD propagation was much more efficient compared to the unmodified Ad5D24 as revealed by ³H-thymidine incorporation and by DNA ethidium bromide staining (Figure 3).

Thus the infectivity advantage conferred by the HI loop modification increased adenovirus propagation in target cells. As this tropism modification would not be anticipated to alter fundamental aspects of the viral replication cycle, this effect was likely achieved exclusively on the basis of infectivity enhancement allowed via routing the virus to CAR-independent pathways.

Increased oncolytic potency of the conditionally replicative adenovirus with expanded tropism. The ultimate goal of this study was to demonstrate the increased lytic potency due to the RGD modification of the fiber. To this end, cells were infected with very low amounts of each virus to allow for multiple cycles of viral replication. Subsequent to this treatment, the amount of cells remaining at 8 days (A549, LNCaP) or 24 days (SKOv3.ip1) post-infection were detected by crystal violet staining. Very few cells were detected after for Ad5-D24RGD infected cells in comparison to cells infected with other viruses, even with wild type Ad5 (Figure 4). These results indicate the enhanced lytic potency of Ad5-D24RGD over the non-modified viruses (Ad5-wt and Ad5-D24), and the modified non-replicative virus (Ad5lucRGD).

(7). KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that prostate carcinoma cells are relatively resistant to adenoviral vector infection based on deficiency of the primary adenovirus receptor, CAR.
- Demonstration that this CAR deficiency in prostate carcinoma cells can be overcome by Ad vectors capable of CAR-independent gene transfer.
- Definition of Ad vectors capable of high efficiency gene delivery to human prostate carcinoma cells.
- Demonstration that infectivity-enhancement maneuvers can augment the oncolytic potency of CRADs for prostate carcinoma cells.

(8). REPORTABLE OUTCOMES

CRAD agents are of exceptional interest currently in the context of cancer therapeutics. We have defined herein a method to dramatically enhance their oncolytic potency. This result will have significance for all investigations currently employing CRAD agents for cancer therapy.

(9). CONCLUSIONS

We have established the key concept that we can augment the oncolytic potency of CRAD agents for carcinoma of the prostate. This has been achieved via modification of the Ad fiber to allow infectivity enhancement of the vector.

Several reasons can be postulated in favor of fiber modifications of replication-competent vectors to achieve extensive tumor transduction. Results from adenoviral-mediated gene delivery to human primary tumors or human tumors in clinical trials have pointed out the limiting amounts of adenoviral receptor CAR in tumors (5,6). This could probably affect the efficacy of replication-competent adenoviruses in similar way that is affecting replication-defective vectors. In theory, the amount of a replication competent virus necessary to achieve a certain level of tumor lysis or transduction is much lower compared to a non-replicating vector due to the viral amplification in the tumor. This

hypothesis has been demonstrated recently (14,15). However, the level of primary infection and intratumoral propagation due to secondary infections will depend on the amount of adenovirus receptor throughout the tumor. Faster infection and spread could have major implications if a neutralizing antiviral immune response is quickly elicited, and restricts further viral spread (16).

The advantage of the RGD modification to increase adenoviral infectivity has been extensively demonstrated in various studies (7,12). Our results are limited to this RGD modification, but other modifications can be considered with the isolation of other tumor-binding peptides (17). Of note, the RGD modification in this study does not preclude the finding of the fiber to CAR and the modified virus can enter the cells through α_v integrins and CAR. Other fiber modifications that ablate CAR-binding would result in truly targeted viruses, but the efficiency of propagation of these viruses will depend on the amount of the targeted receptor in the same way as the propagation of the unmodified virus depends on CAR. This strategy could be very valuable when the population to be targeted is homogeneous, such as endothelial cells of tumors. On the other hand, the specificity achieved at the level of viral replication will dictate how much specificity is needed at the level of receptor binding to obtain a safe virus, and vice versa.

Having thus addressed the infectivity aspect of CRADs, it is now reasonable to endeavor our originally proposed goal of improving CRAD agents via increasing the specificity of tumor-selective replication. In the aggregate, these two maneuvers will dramatically improve the therapeutic index of CRADs for carcinoma of the prostate.

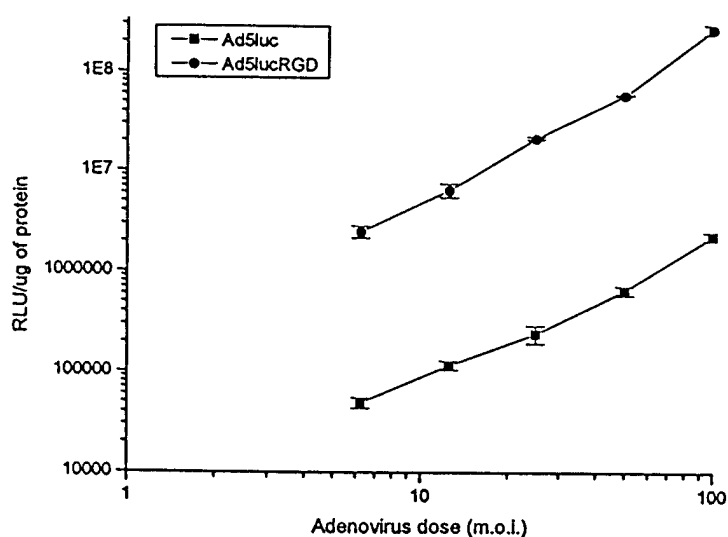
(10). REFERENCES

1. Kirn DH, and McCormick F. Replicating viruses as selective cancer therapeutics. *Mol Med Today* 2:519-527, 1996.
2. Yu DC, Sakamoto GT, and Henderson DR. Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. *Cancer Research* 59:1498-1504, 1999.
3. Rodriguez R, Schuur ER, Lim HY, Henderson GA, Simons JW, and Henderson DR. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Research* 57:2559-2563, 1997.
4. Bischoff JR, Kirn DH, Williams A, Heise C, Horn S, Muna M, Ng L, Nye JA, Sampson-Johannes A, Fattaey A, and McCormick F. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science* 274:373-376, 1996.
5. Li Y, Pong RC, Bergelson JM, Hall MC, Sagalowsky AI, Tseng CP, Wang Z, and Hsieh JT. Loss of adenoviral receptor expression in human bladder cancer cells: a potential impact on the efficacy of gene therapy. *Cancer Research* 59:325-330, 1999.

6. Hemmi S, Geertsen R, Mezzacasa A, Peter I, and Dummer R. The presence of human coxsackievirus and adenovirus receptor is associated with efficient adenovirus-mediated transgene expression in human melanoma cell cultures. *Human Gene Therapy* 9:2363-2373, 1998.
7. Dmitriev I, Krasnykh V, Miller CR, Wang M, Kashentseva E, Mikheeva G, Belousova N, and Curiel DT. An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *Journal of Virology* 72:9706-9713, 1998.
8. Moran E, Zerler B, Harrison TM, and Mathews MB. Identification of separate domains in the adenovirus E1A gene for immortalization activity and the activation of virus early genes. *Molecular Cell Biology* 6:3470-3480, 1986.
9. Egan C, Jelsma TN, Howe JA, Bayley ST, Ferguson B, and Branton PE. Mapping of cellular protein-binding sites on the products of early-region 1A of human adenovirus type 5. *Molecular Cell Biology* 8:3955-3959, 1988.
10. Fueyo J, Gomez-Manzano C, Alemany R, Lee P, McDonell T, Mitlianga P, Shi Y, Yung W, and Kyritsis A. A mutant oncolytic adenovirus targeting the Rb pathway in brain tumors. *Nature Medicine* (Submitted, 1999).
11. Hardy S, Kitamura M, Harris-Stansil T, Dai Y, and Phipps ML. Construction of adenovirus vectors through Cre-lox recombination. *Journal of Virology* 71:1842-1849, 1997.
12. Wickham TJ, Tzeng E, Shears LL, Roelvink PW, Li Y, Lee GM, Brough DE, Lizonova A, and Kovesdi I. Increased *in vitro* and *in vivo* gene transfer by adenovirus vectors containing chimeric fiber proteins. *Journal of Virology* 71:8221-8229, 1997.
13. Vanderwaak T, Wang M, Gomez-Navarro J, Rancourt C, Dmitriev I, Krasnykh V, Barnes M, Siegal G, Alvarez R, and Curiel D. An advanced generation of adenoviral vectors selectively enhances gene transfer for ovarian cancer gene therapy approaches. *Gynecologic Oncology* 74:227-234, 1999.
14. Wildner O, Blaese RM, and Morris JC. Therapy of colon cancer with oncolytic adenovirus is enhanced by the addition of herpes simplex virus-thymidine kinase. *Cancer Research* 59:410-413, 1999.
15. Wildner O, Morris JC, Vahanian NN, Ford Jr H, Ramsey WJ, and Blaese RM. Adenoviral vectors capable of replication improve the efficacy of HSVtk/GCV suicide gene therapy of cancer. *Gene Therapy* 6:57-62, 1999.
16. Russell SJ. Replicating vectors for gene therapy of cancer: risks, limitations, and prospects. *Eur J Cancer* 30A:1165-1171, 1994.
17. Koivunen E, Arap W, Rajotte D, Lahdenranta J, and Pasqualini R. Identification of receptor ligands with phage display peptide libraries. *Journal of Nucleic Medicine* 40:883-888, 1999.

(11). APPENDICES

A.



B.

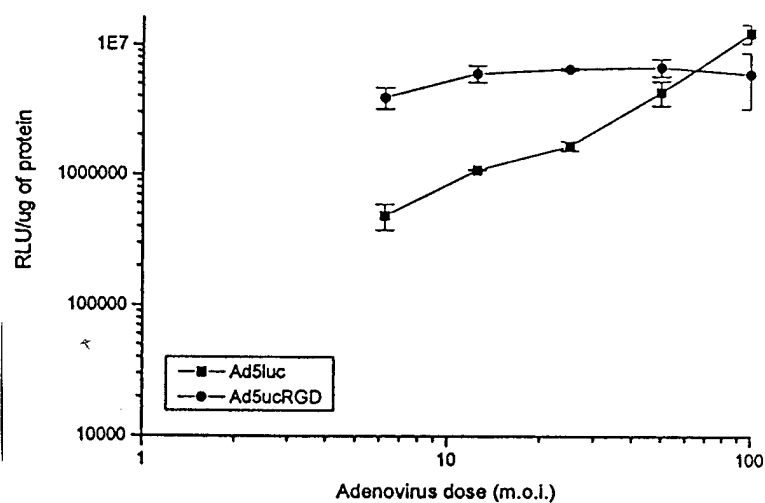


Figure 1: Enhancement of adenovirus infectivity by RGD modification of the fiber. A549 cells (A) and LNCaP cells (B) were transduced with increasing doses of either Ad5luc or Ad5lucRGD. After 18 h, cell transduction was determined by luciferase assay, showing an infectivity advantage of the RGD modified vector over the non-modified one in both cell lines.

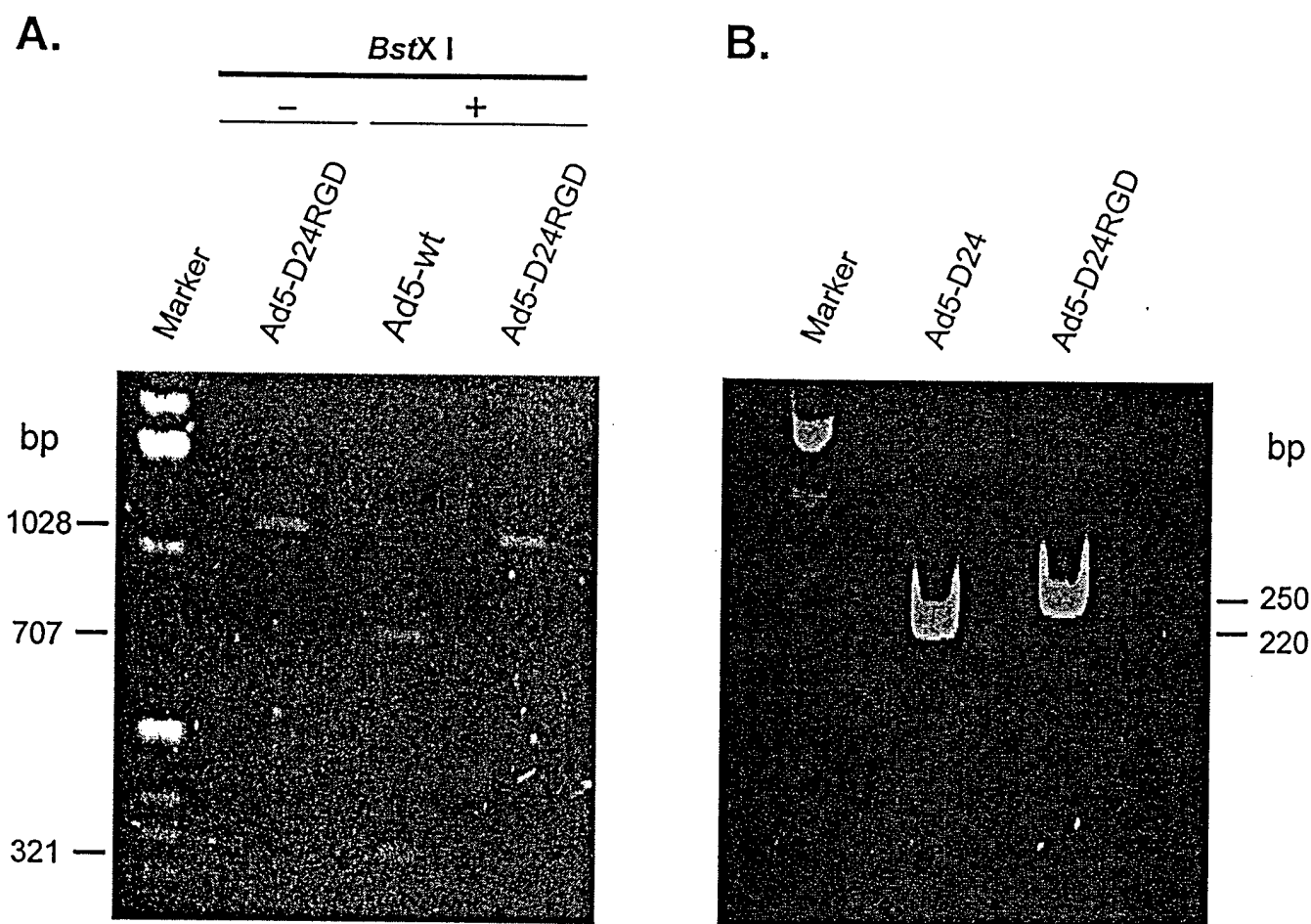


Figure 2: Analysis of adenoviral DNA. (A). Restriction analysis of D24 deletion in Ad5-D24RGD and Ad5-wt. Viral DNA was digested with *Bst*X I. The digested fragments were resolved on a 2% agarose gel containing ethidium bromide (EtBr), and then visualized by UV fluorescence. Uncleaved PCR product indicates the presence of the deletion (B). DNA from Ad5-D24 and Ad5-D24RGD were resolved on a 6% acrylamide gel, stained with EtBr, and visualized by VU light. The bigger size (30 bp) of Ad5-D24RGD indicates the presence of the sequence encoding RGD.

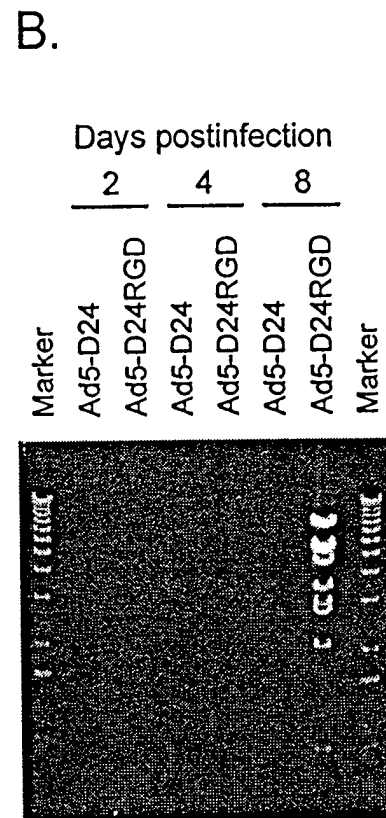
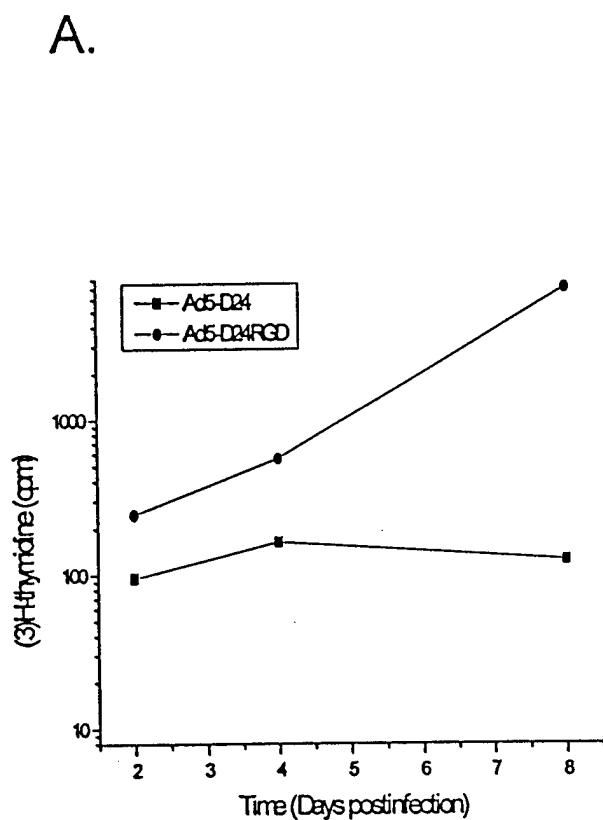


Figure 3: Propagation efficiency of Ad5-D24 versus Ad5-D24RGD. A549 cells were infected with a low dose of Ad5lucRGD, and Ad5-D24, and incubated in supplemented media containing 30 $\mu\text{Ci/mL}$ of ^3H -thymidine during the entire experiment. At the indicated day post-infection, the cells were harvested, and the encapsidated DNA was purified by the spermine-HCl method. (A). DNA corresponding to one million cells was measured by scintillation counting, (B). The same amount of DNA was digested with Hind III, and the fragments were resolved on a 1% agarose gel. Both results indicate that Ad5-D24RGD replication was more efficient compared its unmodified version.

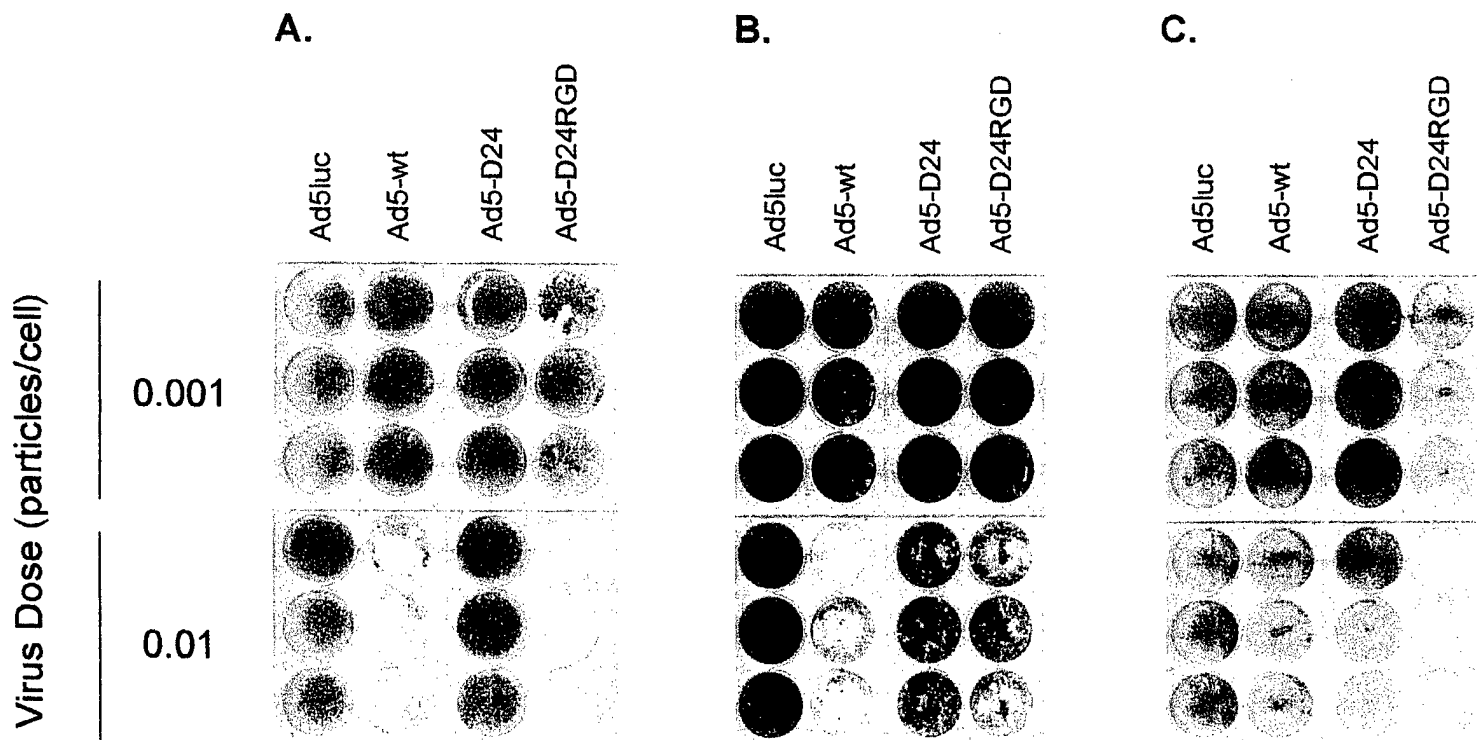


Figure 4: Oncolytic potency of the modified virus. Three tumor cell lines were infected with low doses of Ad5lucRGD, Ad5-wt, Ad5-D24, and Ad5-D24RGD. At 8 days post-infection, A549 (A) and LNCaP (B) cells were fixed and stained with crystal violet. The same process was applied to SKOV3.ip1 (C) 24 days after the infection. In the 3 lines, RGD modified adenovirus showed higher lytic potency than the non-modified counterpart.

A Conditionally Replicative Adenovirus with Enhanced Infectivity Shows Improved Oncolytic Potency

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Running title: Conditionally-Replicative Adenovirus with Enhanced Infectivity

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ABSTRACT

Lack of adenoviral receptor in tumors could limit the use of recently proposed tumor-selective replicative adenoviruses. A double strategy consisting of deletion of the E1A adenoviral gene, and the introduction of an RGD motif in the virus fiber by genetic modification, was used to enhance tumor-specific lysis by replication-competent adenovirus. The double-modified adenovirus showed higher viral DNA replication and virus production, as well as more efficient killing of different tumor cell lines, compared with non-modified adenoviruses. We conclude that the combination of capsid modifications to enhance infectivity, and genomic modifications to achieve tumor-selective replication, yields more potent oncolytic adenoviruses for cancer treatment.

Key Words: Cancer, Gene Therapy, Oncolysis, Virotherapy, Adenovirus, CAR, Integrin

INTRODUCTION

Conditionally-replicative adenoviruses (CRADs) represent a novel and promising approach to treat neoplastic diseases (1). This approach offers several conceptual advantages over conventional gene therapy approaches. First, the replicative property of these agents circumvents the need to achieve quantitative tumor cell transduction. Thus, the intrinsic amplification offered by replicative viruses potentially allows oncolysis with extensive tumor transduction. Secondly, the property of specific replication offers the potential to achieve an improved therapeutic index. On this basis, CRAD-based strategies have been rapidly translated into clinical trials.

Realization of the full utility of CRADs is subservient to their capacity to function as infectious agents in human tumors. After infection, the agent must replicate specifically, propagate laterally, and avoid the host immune response. Strategies to address each of these steps may improve the utility of these agents. To this end, a variety of approaches have been implemented to achieve tumor-specific replication (1). One approach is based on the control of the expression of an essential early viral gene using tumor-specific promoters (2, 3). Another approach is based on deletions made in viral genes encoding proteins that interact with cellular proteins to complete the lytic life cycle (4).

In addition, limitations in the infectivity of tumor cells by adenoviruses can potentially curtail the initial infection and lateral propagation of CRADs. In this regard, human clinical trials with adenovirus have demonstrated relatively inefficient infectivity *in vivo*. This has been related to the scarcity of the primary adenovirus receptor, coxsackie-adenovirus receptor (CAR), on tumor cells. Based on this recognition, it has been proposed that gene delivery via CAR-independent pathways may be required to circumvent this aspect of tumor biology (5, 6).

To address the issue of limited infectivity of tumors by adenoviruses, we have developed methods to enhance infectivity via CAR-independent entry pathways. One approach is based on the genetic modification of the HI loop of the virus fiber (7). A virus containing a fiber modified with an RGD peptide in the HI loop has an expanded tropism because it binds the cells through RGD/integrin interactions in addition to CAR (7). Here, we show that the incorporation of the RGD motif into the fiber of a CRAD enhances its oncolytic potency. Tropism modifications such the introduction of RGD presented here, are thus clearly necessary to overcome limitations in tumor infectivity and to realize the full potential of CRADs.

MATERIALS AND METHODS

Construction of Adenoviruses

Ad5-D24 mutant. Ad5-D24 adenovirus was obtained from Juan Fueyo at M.D. Anderson Cancer Center. This virus contains a 24 nucleotide deletion, from Ad5 bp 923 to 946, corresponding to the amino-acid sequence L₁₂₂TCHEAGF₁₂₉ of E1A protein, known to be necessary for retinoblastoma (Rb) protein binding (8, 9). The tumor-specific replication of this virus is presented elsewhere (10).

RGD modification of Ad5-D24. Ad5lucRGD is an E1-deleted vector containing the recombinant fiber-RGD and expressing the firefly luciferase protein (7). This vector was previously constructed by homologous recombination of the E1 region containing the luciferase gene into plasmid pVK503 that contains the modified fiber. To construct the RGD-modified version of Ad5-D24, we followed a similar procedure. A fragment of DNA encompassing the E1 region

with the D24 deletion was isolated from the plasmid pXC1-D24 originally used to construct Ad5-D24. This E1 fragment was used for homologous recombination with Cla I-digested plasmid pVK503 containing the RGD-fiber (7). After the homologous recombination, the genome of the new virus was released from the plasmid backbone by digestion with Pac I enzyme. The plasmid obtained as a result of this DNA recombination was then utilized for transfection of 293 cells to rescue Ad5-D24RGD. The RGD presence in the Ad5-D24RGD and Ad5lucRGD and was confirmed by PCR employing fiber primers FiberUp (5'-CAAACGCTGTTGGATTTATG-3') and FiberDown (5'-GTGTAAGAGGATGTGGCAAAT-3'). The D24 deletion was routinely analyzed by PCR with primers E1a-1 (5'-ATTACCGAAGAAATGGCCGC-3') and E1a-2 (5'-CCATTTAACACGCCATGCA-3'), followed by BstX I digestion. Uncleaved PCR product indicated the presence of the deletion.

Cell Lines and Culture

Three cell lines were employed: the human lung adenocarcinoma cell line A549 (obtained from ATCC), the human prostate cancer cell line LNCaP (from ATCC), and the human ovarian cancer cell line SKOv3.ip1 (generously provided by Janet Price, M.D. Anderson Cancer Center, Houston, TX). The cells were cultured in a humidified chamber at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% of heat-inactivated fetal bovine serum, 100 I.U./mL penicillin, and 100 ug/mL streptomycin. The adenovirus-transformed human embryonic kidney cell line 293 (from ATCC) was cultured in the same media and used for virus titration.

RGD-mediated infectivity enhancement

The cell lines were cultured at 90% confluence in 6 well plates and infected with Ad5luc or Ad5lucRGD at viral doses ranging from 1 to 100 plaque forming units (pfu)/cell. After 2 h, the cells were washed with PBS and fresh media was added. All the groups were harvested at 18 h postinfection, lysed, and luciferase expression was quantitated using Promega's luciferase assay system following manufacturer recommendations (Promega, Madison, WI).

Virus DNA replication

Cells were cultured at 90% confluence in 6 well plates and infected with Ad5-wt, Ad5-D24, Ad5-D24RGD, and Ad5lucRGD at 0.01 particles/cell. After 2 h, the cells were washed and media with ^3H -thymidine (30 $\mu\text{Ci/mL}$) was added. Attached and detached cells were harvested at 8 days postinfection and encapsidated viral DNA was purified using the spermine-HCl method (11). One half of the total purified viral DNA (corresponding to 10^6 cells) was then digested with Hind III, and run in 1% agarose gel. The other half was used to measure the amount of radioactivity in scintillation solution.

Adenovirus Yield Assay

Cell monolayers at 90% confluence on 6 well plates were infected with Ad5-wt, Ad5-D24, Ad5-D24RGD, and Ad5lucRGD, at doses of 0.001 and 0.01 particles/cell for 2 h. The cells were then washed thoroughly with PBS to remove all non-adsorbed virus, and medium was added. At 2, 4, and 8 days the medium was harvested, and the titer of each type of adenovirus was quantified using the plaque assay with 293 cells as targets.

Oncolysis assay

Cells were seeded in 6 well plates and infected with the four types of adenovirus when confluence reached 90%. Eight days postinfection, A549 and LNCaP cells were washed with PBS and fixed with fresh buffered formaldehyde for 10 min. Fixed cells were stained with 1% crystal violet solution for 1 h and then rinsed and dried. For SKOV3.ip1 longer times of incubation (24 days post-infection) were used before the crystal violet staining.

RESULTS

Enhancement of virus infectivity mediated by the RGD peptide incorporated into the HI loop to the fiber knob

Incorporation of RGD into adenoviral fiber has been shown to increase adenoviral vector infectivity in a variety of cell lines and primary tumors (7, 12). We have chosen three cell lines representative of tumor types that we are interested in: A549 lung adenocarcinoma, LNCaP prostate carcinoma, and SKOV3.ip1 ovarian carcinoma. A four-fold increase in transduction mediated by RGD in SKOV3.ip1 has been reported previously (13). The increase in transduction resulting from the modification of the fiber with an RGD sequence in A549 cells and LNCaP cells was determined by infection with E1-substituted vectors expressing luciferase containing the wild type fiber or the RGD-modified fiber. In both cell lines the RGD modified vector showed an infectivity advantage over the non-modified counterpart (Figure 1). The major differences were observed in A549 cells with a 100x increase in transduction, followed by

LNCaP with a 10x increase. In LNCaP the major differences were observed at lower multiplicities of infection indicating that the CAR mediated pathway was quickly saturated.

Propagation advantage of an RGD modified conditionally replicative adenovirus

To take advantage of the increased infectivity conferred by the RGD modification in a replication-competent context, we chose an adenovirus with a deletion in the retinoblastoma-binding site of E1A. The characterization of this virus in terms of tumor-specific replication will be presented elsewhere (10). The D24 deletion of E1A, and the RGD insertion in the fiber, were combined into a unique viral genome by homologous recombination in bacteria. The resulting virus, Ad5-D24RGD, was propagated efficiently in a variety of cell lines. The presence of the D24 mutation in E1A and the RGD insertion in the fiber was analyzed by PCR (Figure 2). Of note, we have not observed the outcome of adenoviruses with wild-type E1 or wild-type fiber throughout the propagation of Ad5-D24RGD concordant with the lack of endogenous adenoviral sequences in A549, cells that we regularly used to propagate this virus.

After structural confirmation, Ad5-D24RGD, Ad5D24, Ad5-wt, and Ad5lucRGD were compared in their ability to replicate in the three cell lines studied. Cell monolayers were infected with a small amount of each virus (0.01 m.o.i.) in the presence of ^3H -thymidine. During an 8-day incubation period, the encapsidated viral DNA was isolated at days 2, 4, and 8. Viral DNA corresponding to one million cells was analyzed by gel electrophoresis and quantified by scintillation counting. Ad5-D24RGD propagation was much more efficient compared to the unmodified Ad5D24 as revealed by ^3H -thymidine incorporation and by DNA ethidium bromide staining (Figure 3). Thus the infectivity advantage conferred by the HI loop modification increased adenovirus propagation in target cells. As this tropism modification would not be

anticipated to alter fundamental aspects of the viral replication cycle, this effect was likely achieved exclusively on the basis of infectivity enhancement allowed via routing the virus to CAR-independent pathways.

Increased oncolytic potency of the conditionally replicative adenovirus with expanded tropism

The ultimate goal of this study was to demonstrate the increased lytic potency due to the RGD modification of the fiber. To this end, cells were infected with very low amounts of each virus to allow for multiple cycles of viral replication. Subsequent to this treatment, the amount of cells remaining at 8 days (A549, LNCaP) or 24 days (SKOv3.ip1) post-infection were detected by crystal violet staining. Very few cells were detected after for Ad5-D24RGD infected cells in comparison to cells infected with other viruses, even with wild type Ad5 (Figure 4). These results indicate the enhanced lytic potency of Ad5-D24RGD over the non-modified viruses (Ad5-wt and Ad5-D24), and the modified non-replicative virus (Ad5lucRGD).

DISCUSSION

Several reasons can be postulated in favor of fiber modifications of replication-competent vectors to achieve extensive tumor transduction. Results from adenoviral-mediated gene delivery to human primary tumors or human tumors in clinical trials have pointed out the limiting amounts of adenoviral receptor CAR in tumors (5, 6). This could probably affect the efficacy of replication-competent adenoviruses in a similar way that is affecting replication-defective

vectors. In theory, the amount of a replication competent virus necessary to achieve a certain level of tumor lysis or transduction is much lower compared to a non-replicating vector due to the viral amplification in the tumor. This hypothesis has been demonstrated recently (14, 15). However, the level of primary infection and intratumoral propagation due to secondary infections will depend on the amount of adenovirus receptor throughout the tumor. Faster infection and spread could have major implications if a neutralizing antiviral immune response is quickly elicited, and restricts further viral spread (16).

The advantage of the RGD modification to increase adenoviral infectivity has been extensively demonstrated in various studies (7, 12). Our results are limited to this RGD modification, but other modifications can be considered with the isolation of other tumor-binding peptides (17). Of note, the RGD modification in this study does not preclude the binding of the fiber to CAR and the modified virus can enter the cells through α_v integrins and CAR. Other fiber modifications that ablate CAR-binding would result in truly targeted viruses, but the efficiency of propagation of these viruses will depend on the amount of the targeted receptor in the same way as the propagation of the unmodified virus depends on CAR. This strategy could be very valuable when the population to be targeted is homogeneous, such as endothelial cells of tumors. On the other hand, the specificity achieved at the level of viral replication will dictate how much specificity is needed at the level of receptor binding to obtain a safe virus, and vice versa.

Truly targeted conditionally replication-competent adenoviral vectors have theoretical attributes that could make them effective via systemic administration: low toxicity (due the lack of adsorption and replicate in normal cells) and low effective dose (due to their amplification).

Whether these vectors have enough targeting/amplification potency to be efficacious via systemic administration remains to be shown. Vector clearance by liver macrophages is a major obstacle that has to be overcome. This can be attempted with targeting or other strategies that change the physico-chemical properties of the virion such as PEGylation (18). Immune responses will play an important role in the final outcome of oncolytic virotherapy, an ideal scenario would favor a response that can destroy tumor cells*still allowing viral spread. The manipulation of the immune response against adenovirus towards a TH1 type could lead in this direction (19). The emerging picture is that of a targeted adenoviral vector that remains in circulation searching for tumors and that engages in an amplifying killing cascade when it finds a tumor.

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REFERENCES

1. Kim, D. H. and McCormick, F. Replicating viruses as selective cancer therapeutics, *Mol Med Today*. 2: 519-27, 1996.
2. Yu, D. C., Sakamoto, G. T., and Henderson, D. R. Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy, *Cancer Res*. 59: 1498-504, 1999.
3. Rodriguez, R., Schuur, E. R., Lim, H. Y., Henderson, G. A., Simons, J. W., and Henderson, D. R. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells, *Cancer Res*. 57: 2559-63, 1997.
4. Bischoff, J. R., Kim, D. H., Williams, A., Heise, C., Horn, S., Muna, M., Ng, L., Nye, J. A., Sampson-Johannes, A., Fattaey, A., and McCormick, F. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells, *Science*. 274: 373-6, 1996.
5. Li, Y., Pong, R. C., Bergelson, J. M., Hall, M. C., Sagalowsky, A. I., Tseng, C. P., Wang, Z., and Hsieh, J. T. Loss of adenoviral receptor expression in human bladder cancer cells: a potential impact on the efficacy of gene therapy, *Cancer Res*. 59: 325-30, 1999.
6. Hemmi, S., Geertsen, R., Mezzacasa, A., Peter, I., and Dummer, R. The presence of human coxsackievirus and adenovirus receptor is associated with efficient adenovirus-mediated transgene expression in human melanoma cell cultures, *Hum Gene Ther*. 9: 2363-73, 1998.
7. Dmitriev, I., Krasnykh, V., Miller, C. R., Wang, M., Kashentseva, E., Mikheeva, G., Belousova, N., and Curiel, D. T. An adenovirus vector with genetically modified fibers

demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism, *J Virol.* 72: 9706-13, 1998.

8. Moran, E., Zerler, B., Harrison, T. M., and Mathews, M. B. Identification of separate domains in the adenovirus E1A gene for immortalization activity and the activation of virus early genes, *Mol Cell Biol.* 6: 3470-80, 1986.

9. Egan, C., Jelsma, T. N., Howe, J. A., Bayley, S. T., Ferguson, B., and Branton, P. E. Mapping of cellular protein-binding sites on the products of early- region 1A of human adenovirus type 5, *Mol Cell Biol.* 8: 3955-9, 1988.

10. Fueyo, J., Gomez-Manzano, C., Alemany, R., Lee, P., McDonell, T., Mitlianga, P., Shi, Y., Yung, W., and Kyritsis, A. A mutant oncolytic adenovirus targeting the Rb pathway in brain tumors, Submitted *Nat Med*, 1999.

11. Hardy, S., Kitamura, M., Harris-Stansil, T., Dai, Y., and Phipps, M. L. Construction of adenovirus vectors through Cre-lox recombination, *J Virol.* 71: 1842-9, 1997.

12. Wickham, T. J., Tzeng, E., Shears LL, n., Roelvink, P. W., Li, Y., Lee, G. M., Brough, D. E., Lizonova, A., and Kovesdi, I. Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins, *J Virol.* 71: 8221-9, 1997.

13. Vanderkwaak, T., Wang, M., Gomez-Navarro, J., Rancourt, C., Dmitriev, I., Krasnykh, V., Barnes, M., Siegal, G., Alvarez, R., and Curiel, D. An advanced generation of adenoviral vectors selectively enhances gene transfer for ovarian cancer gene therapy approaches., *Gynecol Oncol*, In Press. 1999.

14. Wildner, O., Blaese, R. M., and Morris, J. C. Therapy of colon cancer with oncolytic adenovirus is enhanced by the addition of herpes simplex virus-thymidine kinase, *Cancer Res.* 59: 410-3, 1999.

15. Wildner, O., Morris, J. C., Vahanian, N. N., Ford, H., Jr., Ramsey, W. J., and Blaese, R. M. Adenoviral vectors capable of replication improve the efficacy of HSVtk/GCV suicide gene therapy of cancer, *Gene Ther.* *6*: 57-62, 1999.
16. Russell, S. J. Replicating vectors for gene therapy of cancer: risks, limitations and prospects, *Eur J Cancer.* *30A*: 1165-71, 1994.
17. Koivunen, E., Arap, W., Rajotte, D., Lahdenranta, J., and Pasqualini, R. Identification of receptor ligands with phage display peptide libraries, *J Nucl Med.* *40*: 883-8, 1999.
18. O'Riordan, C., Lachapelle, A., Delgado, C., Parkes, V., Wadsworth, S., Smith, A., and Francis, G. PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo., *Hum Gene Ther.* *10*: 1349-1358, 1999.
19. Yang, Y., Trinchieri, G., and Wilson, J. M. Recombinant IL-12 prevents formation of blocking IgA antibodies to recombinant adenovirus and allows repeated gene therapy to mouse lung, *Nat Med.* *1*: 890-3, 1995.

FIGURE LEGENDS

Figure 1. Enhancement of adenovirus infectivity by RGD modification of the fiber. A549 cells (A) and LNCaP cells (B) were transduced with increasing doses of either Ad5luc or Ad5lucRGD. After 18 h, cell transduction was determined by luciferase assay, showing an infectivity advantage of the RGD modified vector over the non-modified one in both cell lines.

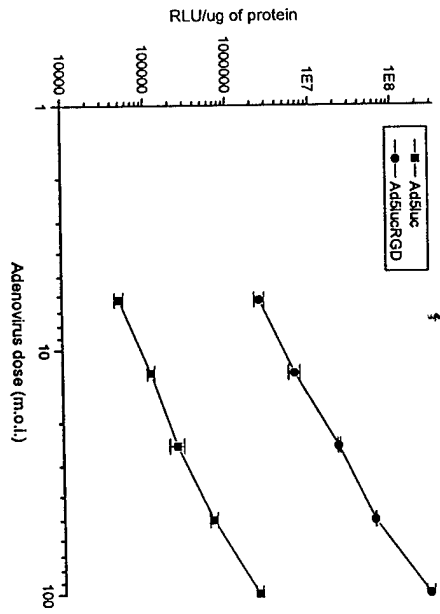
Figure 2. Analysis of adenoviral DNA. (A) Restriction analysis of D24 deletion in AD5-D24RGD and Ad5-wt. Viral DNA was digested with *Bst*X I. The digested fragments were resolved on a 2% agarose gel containing ethidium bromide (EtBr), and then visualized by UV fluorescence. Uncleaved PCR product indicates the presence of the deletion. (B) DNA from Ad5-D24 and Ad5-D24RGD were resolved on a 6% acrylamide gel, stained with EtBr, and visualized by UV light. The bigger size (30 bp) of Ad5-D24RGD indicates the presence of the sequence encoding RGD.

Figure 3. Propagation efficiency of Ad5-D24 versus Ad5-D24RGD. A549 cells were infected with a low dose of Ad5lucRGD, and Ad5-D24, and incubated in supplemented media containing 30 uCi/mL of ^3H -thymidine during the entire experiment. At the indicated day post-infection, the cells were harvested, and the encapsidated DNA was purified by the

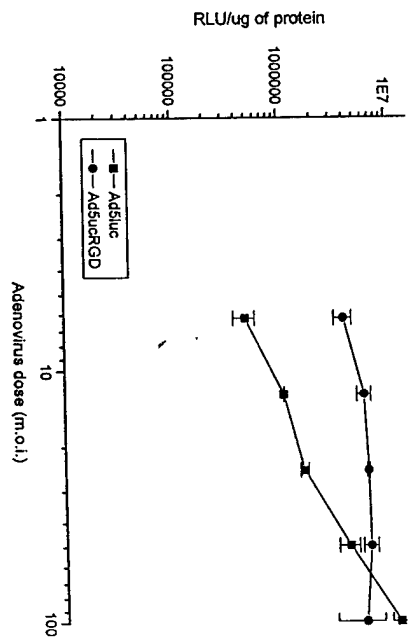
spermine-HCl method. A) DNA corresponding to one million cells was measured by scintillation counting. B) The same amount of DNA was digested with Hind III, and the fragments were resolved on a 1% agarose gel. Both results indicate that Ad5-D24RGD replication was more efficient compared its unmodified version.

Figure 4. Oncolytic potency of the modified virus. Three tumor cell lines were infected with low doses of Ad5lucRGD, Ad5-wt, Ad5-D24, and Ad5-D24RGD. At 8 days post-infection, A549 (A) and LNCaP (B) cells were fixed and stained with crystal violet. The same process was applied to SKOv3.ip1 (C) 24 days after the infection. In the 3 lines, RGD modified adenovirus showed higher lytic potency than the non-modified counterpart.

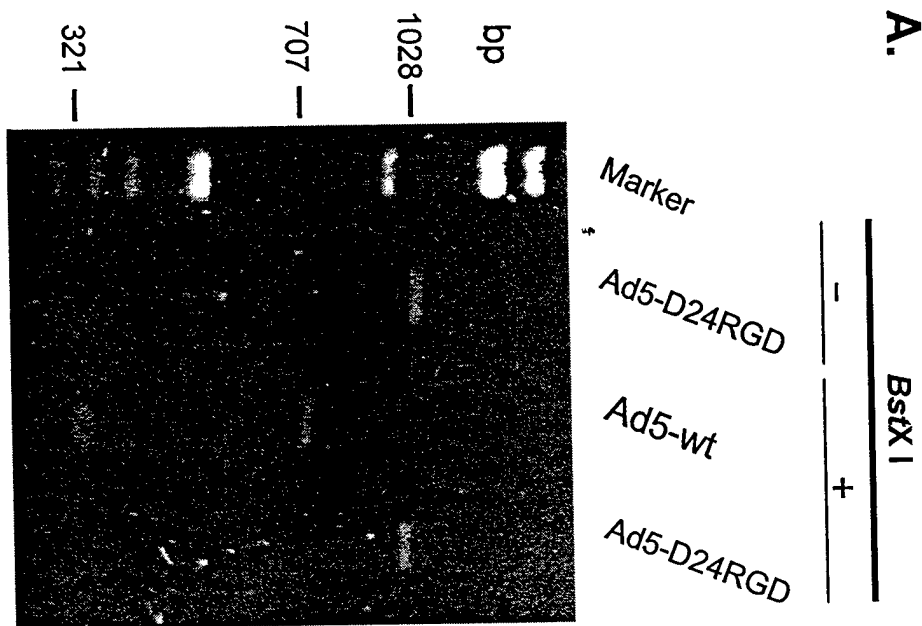
A.



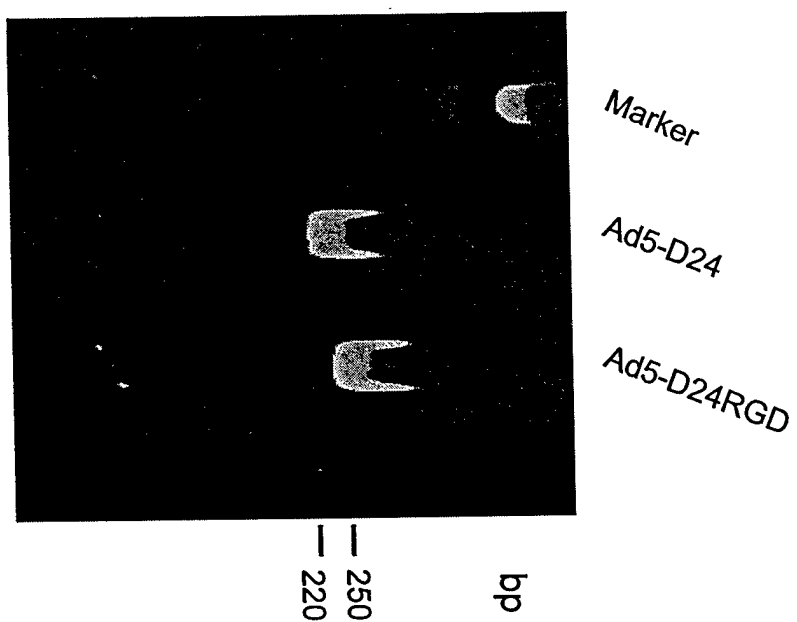
B.



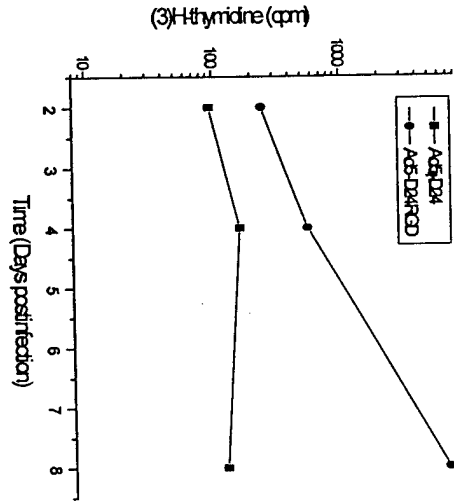
A.



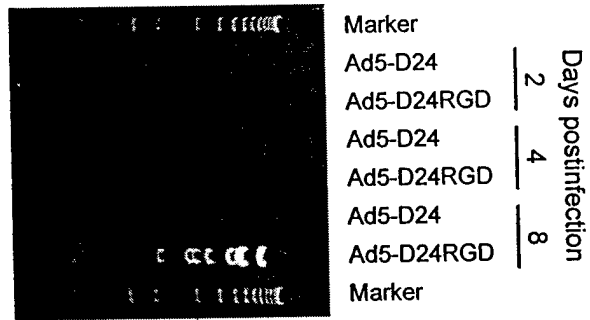
B.



A.



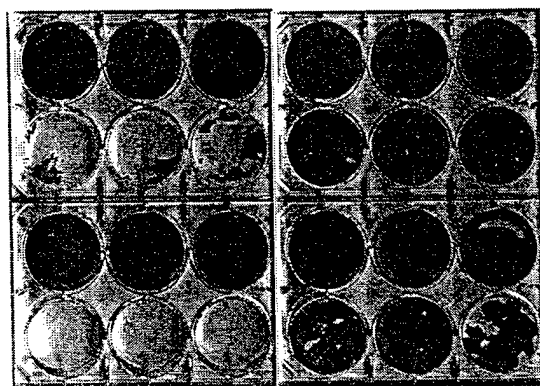
B.



Virus Dose (particles/cell)

0.01

0.001



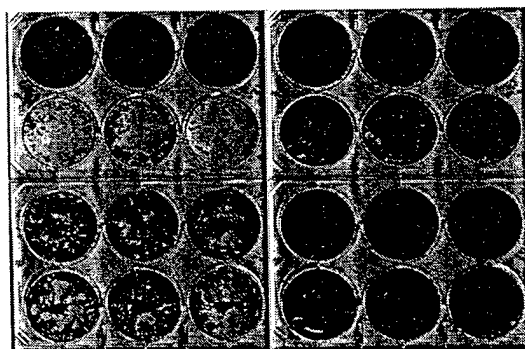
Ad5luc

Ad5-wt

Ad5-D24

Ad5-D24RGD

A.



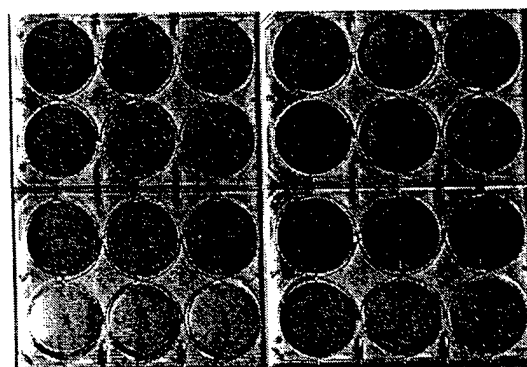
Ad5luc

Ad5-wt

Ad5-D24

Ad5-D24RGD

B.



Ad5luc

Ad5-wt

Ad5-D24

Ad5-D24RGD

C.

**INFECTIVITY-ENHANCED CONDITIONALLY-REPLICATIVE
ADENOVIRUS AND USES THEREOF**

5

10

BACKGROUND OF THE INVENTION

Federal Funding Legend

15 This invention was produced in part using funds obtained
through a grant from the National Institutes of Health. Consequently,
the federal government has certain rights in this invention.

Field of the Invention

20 The present invention relates generally to adenoviral
vectors and adenoviral gene therapy. More specifically, the present

invention relates to an infectivity-enhanced conditionally replicative adenovirus.

Description of the Related Art

5 Surgery, chemotherapy and radiotherapy constitute the conventional therapies in clinical use to treat cancer. These therapies have produced a high rate of cure in early-stage cancer, but most late-stage cancers remain incurable because they cannot be resected or the dose of radiation or chemotherapy administered is limited by
10 toxicity to normal tissues. An alternative promising approach is the transfer of genetic material to tumor or normal cells as a new therapy itself or to increase the therapeutic index of the existing conventional therapies [1]. In this regard, three main strategies have been developed to accomplish cancer gene therapy: potentiating
15 immune responses against tumors, eliciting direct toxicity to tumors, and compensating the molecular lesions of tumor cells [2].

To achieve the high level of gene transfer required in most cancer gene therapy applications, several viral and non-viral vectors have been designed [13]. Adenoviral vectors have been used
20 preferentially over other viral and non-viral vectors for several reasons, including infectivity of epithelial cells, high titers, *in vivo*

stability, high levels of expression of the transgene, gene-carrying capability, expression in non-dividing cells, and lack of integration of the virus into the genome. In most of the adenoviral vectors used in cancer gene therapy, the transgene substitutes for the early 1 region (E1) of the virus. The E1 region contains the adenoviral genes expressed first in the infectious stage and controls expression of the other viral genes. The early region 3 (E3) gene codes for proteins that block a host's immune response to viral-infected cells and is also usually deleted in vectors used for cancer gene therapy, particularly in immunopotentiating strategies.

E1-substituted, E3-deleted vectors can carry up to 8 kb of non-viral DNA, which is sufficient for most gene therapy applications. E1-substituted, E3-deleted vectors are propagated in packaging cell lines that transcomplement their E1-defectiveness, with production yields of up to 10,000 virion particles per infected cell, depending upon the transgene and its level of expression in the packaging cell. Not all of the viral particles are able to transduce cells or to replicate in the packaging cell line, so bioactivity of a particular vector has been defined as the ratio of functional particles to total particles. This bioactivity varies from 1/10 to 1/1000, depending not only upon the vector, but also upon the methods of purification and

quantification [15]. The titer used is the concentration of functional particles, which can be as high as 10^{12} per milliliter.

One problem encountered when propagating these vectors to high titers is the recombination of vector sequences with the E1 sequences present in the packaging cell line, thereby producing replication-competent adenoviruses (RCA). This problem has been solved by using packaging cell lines where the E1 gene does not overlap with the vector sequences [16].

The current generation of adenoviral vectors are limited in their use for cancer gene therapy, primarily for three reasons: (1) the vectors are cleared by the reticuloendothelial system, (2) they are immunogenic and/or (3) they infect normal cells. The problem of filtration by the reticuloendothelial system cells, such as macrophages of the spleen or Kupffer cells of the liver, affects adenoviral vectors as well as other viral and non-viral vectors and limits their utility in intravascular administration [19]. The early and late viral genes that remain in E1-E3 deleted vectors may also be expressed at low, but sufficient enough levels such that the transduced cells are recognized and lysed by the activated cytotoxic T lymphocytes. Additionally, a higher viral dose must be injected to reach the entire tumor before a neutralizing immune response

develops. The major limitation then becomes the amount of vector that can be safely administered, which will depend upon the capacity of the vector to affect tumor cells without affecting normal cells.

The limitations of adenoviral vectors at the level of infectivity is two-fold. On the one hand, human clinical trials with adenoviral vectors have demonstrated relatively inefficient gene transfer *in vivo*. This has been related to the paucity of the primary adenovirus receptor, coxsackie-adenovirus receptor (CAR), on tumor cells relative to their cell line counterparts [20-23]. On this basis, it has been proposed that gene delivery via CAR-independent pathways may be required to circumvent this key aspect of tumor biology. On the other hand, adenoviral vectors efficiently infect normal cells of many epithelia. This results in the expression of the transgene in normal tissue cells with the consequent adverse effects. This problem has been addressed by targeting adenoviral vectors to tumor cells at the level of receptor interaction and transgene transcription.

Targeting adenoviral vectors to new receptors has been achieved by using conjugates of antibodies and ligands, in which the antibody portion of the conjugate blocks the interaction of the fiber with the CAR receptor and the ligand portion provides binding for a

novel receptor [20]. Receptor targeting has also been achieved by genetic modification of the fiber [23-26]. Transcriptional targeting of adenoviral vectors has further been demonstrated using tumor-antigen promoters or tissue-specific promoters to control the expression of the transgene [27]. However, these promoters can lose their specificity when inserted in the viral genome and, depending upon the level of toxicity of the transgene, even low levels of expression can be detrimental to normal cells. Thus, for cancer gene therapy, the major issues limiting the utility of adenoviral vectors are the efficiency and specificity of the transduction.

The major limitation found in the use of adenoviral vectors in the clinical setting is the number of tumor cells that remain unaffected by the transgene. A vector that propagates specifically in tumor cells, results in lysis and subsequently allows for transduction of neighbor cells by newly produced virions will increase the number of tumor cells affected by the transgene [28]. A good replicative vector should be weakly pathogenic or non-pathogenic to humans and should be tumor-selective [29]. Efforts have been aimed at improving the safety of replication-competent adenoviruses with the goal of being able to administer much higher doses. One strategy is to transcomplement the E1 defect with an E1-

expression plasmid conjugated into the vector capsid [31], which allows a single round of replication thereby producing a new E1-substituted vector with the ability of local amplification and subsequent gene transduction.

5 Other strategies are designed to obtain vectors that replicate continuously and whose progeny are also able to replicate, but are incapable of propagating in normal cells. In this regard, two approaches has been described that render adenovirus propagation selective for tumor cells: (1) deletions, and (2) promoter regulation
10 [30]. Adenoviral mutants unable to inactivate p53 propagate poorly in cells expressing p53 but efficiently in tumor cells where p53 is already inactive. Based upon this strategy, an adenovirus mutant in which the E1b-55k viral protein was deleted and was unable to bind to p53 was effective in eliminating tumors in preclinical models and
15 is in clinical trials [32]. Controlling viral replication by substituting a viral promoter, such as the E1a promoter, with a tumor associated-antigen promoter, such as the alpha-fetoprotein promoter or the prostate antigen promoter, has been demonstrated [33], and specific lysis of tumors transfected with an adenovirus vector expressing
20 either of the above-mentioned promoters was demonstrated in murine models.

Both approaches have limitations, however. The fact that other viral proteins besides E1b 55K also interact with p53, and because p53 can be necessary for the active release of virus in the later stages of infection may affect the specificity of the vector [37,38]. Another caveat results from using E1a as the only controlled viral gene since E1a-like activity has been found in many tumor cell lines [14,40]. Furthermore, the actual specificity of the above-mentioned promoters for cancer cells, and the fact that promoters inserted in the viral genome can lose their expression specificity, are factors that hindered clinical applications of this approach [39].

Therefore, new methods are clearly needed to achieve more selective therapeutic effects of replication-competent adenoviruses. For these vectors, in parallel to what has been achieved with non-replicative vectors, modification of viral tropism could enhance tumor transduction and tumor selectivity at the level of cell entry, and in this way, realize the full potential of replicative vectors for cancer gene therapy.

The prior art is deficient in adenoviral vectors that are specific for a particular cell type (*i.e.*, do not infect other cell types) and that replicate with high efficiency in only those particular cell

types. The present invention fulfills this long-standing need and desire in the art.

5

SUMMARY OF THE INVENTION

Adenoviral vectors have been widely employed in cancer gene therapy. Their high titers, structural stability, broad infectivity, high levels of transgene expression, and lack of integration have contributed to the utility of this vector. In this regard, adenoviral vectors has been used to transfer a variety of genes to treat cancer such as cytokines, tumor suppresser genes, pro-drug converting genes, antisense RNAs and ribozymes to inhibit the expression of oncogenes, antiangiogenic genes, etc. Despite the promise of adenoviral vectors, results from experimental models and clinical trials have been less than optimal.

Within this context, several specific limitations have been identified. One limitation lies in the poor infectability of primary tumors due to low levels of the primary adenovirus receptor CAR. A second limitation that particularly affects the efficiency of replicative vectors is related to the lack of tumor-specific replication achieved

using promoters or mutations. The present invention describes methods to increase adenovirus infectivity based upon modification of the virus tropism. The present invention demonstrates that modification of the adenovirus fiber by genetic manipulation increases infectivity of primary tumors several orders of magnitude due to CAR-independent gene transfer. In addition, selective replication in tumors is described herein, and represents a safe and effective means to lyse and transduce tumors. The present invention further describes a strategy based upon control of the expression of one or more essential early viral genes using tumor-specific promoters.

It is a goal of the present invention to improve the infectivity and specificity of conditional replicative vectors, thereby improving their therapeutic utility and efficacy.

One object of the present invention is to provide adenoviral vectors that possess enhanced infectivity to a specific cell type (*i.e.*, that are not limited to CAR-dependent cell entry) and that replicate with high efficiency in only those cell types.

In an embodiment of the present invention, there is provided an infectivity-enhanced conditionally-replicative adenovirus. This adenovirus possesses enhanced infectivity towards

a specific cell type, which is accomplished by a modification to an HI loop of a fiber knob from the adenovirus. Additionally, the adenovirus has at least one conditionally regulated early gene, such that replication of the adenovirus is limited to the specific cell type.

5 In yet another embodiment of the present invention, there is provided a method of enhanced-infectivity conditionally-replicative adenoviral gene therapy in an individual in need of such treatment. This method comprises the steps of: administering to an individual a therapeutic dose of an infectivity-enhanced
10 conditionally-replicative adenovirus. This adenovirus possesses enhanced infectivity towards a specific cell type, which is accomplished by a modification to an HI loop of a fiber knob from the adenovirus. The adenovirus also has at least one conditionally regulated early gene, such that replication of the adenovirus is
15 limited to the specific cell type.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention. These embodiments are given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

The appended drawings have been included herein so that the above-recited features, advantages and objects of the invention will become clear and can be understood in detail. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and should not be considered to limit the scope of the invention.

Figure 1 shows that an anti-knob Fab-FGF2 conjugate enhances cell transduction. **Figure 1A** shows that AdCMVluc (5×10^7 pfu) was preincubated with 1.44 μg of Fab or 1.94 μg of Fab-FGF2. SKOV3 cells (24,000 cells per well in 24-well plates) were infected with control vector or with the vector complexes (MOI of 50). Inhibition was performed by adding a polyclonal anti-FGF2 to the complex before infection. Luciferase activity in cell lysates was assayed 24 h after infection. The mean of triplicate experiments is shown. **Figure 1B** shows that AdCMVLacZ was complexed with Fab-FGF2 conjugate as in **Figure 1A**. SKOV3 cell were infected with control vector (a, c) or complexed vector (b, d) at MOI of 5 (a, b) or 50 (c, d) and stained with X-gal 24 h after infection.

Figure 2 shows that Fab-FGF2 retargeting augments *in vivo* therapeutic benefit of the AdCMVHSV-TK vector. Five days after i.p. inoculation of 2×10^7 SKOV3 cell in SCID mice, 2×10^8 or 2×10^9 pfu of AdCMVTK alone or complexed with FGF2 were injected i.p. 5 Forty-eight h later, half of the mice were treated with GCV (50 mg/kg body weight) for 14 days. Survival was monitored daily.

Figure 3 shows the HI loop of the fiber as a domain to insert ligand for retargeting adenoviruses. Figure 3A shows the knob trimer viewed along the three-fold symmetry axis. 10 (Reproduced from Xia et al. [42]). Figure 3B shows the localization of targeting ligands within the fiber molecule.

Figure 4 shows adenovirus-mediated gene transfer to various human cell lines. 293 (Figure 4A), human vascular endothelial cells (HUVEC) (Figure 4B) or Rhabdomyosarcoma (RD) 15 (Figure 4C) cells preincubated for 10 min at room temperature in medium containing recombinant Ad5 fiber knob at 100 μ g/ml were then exposed for 30 min at room temperature to AdCMVLuc or Ad5lucRGD in DMEM/F12 at 1, 10 or 100 pfu/cell. The unbound virus was aspirated and complete medium was added. After 20 incubation at 37°C for 30 hours, the cells were lysed and the luciferase activity in relative light units (rlu) was determined.

Background luciferase activities detected in mock infected cells were 261, 223, and 163 rlu for 293, HUVEC and RD cells, respectively. These activities were subtracted from all readings obtained with the corresponding cell line. Each point represents the mean of three
5 determinations \pm SD.

Figure 5 shows a comparison of the gene transfer efficiencies to cultured ovarian cancer cells mediated by AdCMVLuc and Ad5lucRGD. Human ovarian cancer cells SKOV3.ip1 (**Figure 5A**) and OV-4 (**Figure 5B**) were transduced with AdCMVLuc or
10 Ad5lucRGD at an MOI of 1 or 10 pfu/cell essentially as described in **Figure 4** for 293, HUVEC and RD cells. Recombinant Ad5 fiber knob protein was added to cells prior to infection with the virus. Each data point is the average of three independent measurements obtained in one experiment.

Figure 6 shows transduction of primary cells isolated from ascites obtained from ovarian cancer patients. Cells isolated from ascites of two (**Figure 6A and B**) ovarian cancer patients were transduced with AdCMVLuc or Ad5lucRGD at MOI of 1 or 10 in the presence or absence of blocking Ad5 fiber knob protein. The data
20 points represent the mean of three independent determinations.

Figure 7 shows a comparison of expression of luciferase achieved with the RGD-modified vector, AdRGDluc versus the non-modified vector AdCMVluc. For each cell line, 25,000 cells were infected at different MOIs and the luciferase expression was measured 36 h after infection. The mean value of three wells is shown.

Figure 8 shows luciferase expression levels achieved with the RGD-modified vector, AdRGDluc, versus the non-modified vector, AdCMVluc, depending on the adsorption time. A549 lung adenocarcinoma cells (10^5 /well) were incubated with an MOI of 100 pfu/cell during different times (a larger amount of cells and a higher MOI were used relative to the previous experiment in order to achieve detectable expression at short adsorption times). After the adsorption time, the cells were washed three times with PBS and complete medium was added. Luciferase was measure 36 h after infection. The mean value of three wells is shown.

Figure 9 shows a conceptual representation of the conditional replication enablement system for adenovirus. The initial introduction of recombinant virus into the tumor mass infects the cells shown as circles. The replication enabling plasmid converts

these cells in vector-producing cells. The produced vector can infect adjacent cells (arrows).

Figure 10 shows functional analysis of pE1FR. LS174T cells were cotransduced with the plasmid indicated in the abscissa as a liposomic complex (0.5 μ g DNA/1.0 μ g DOTAP:DOPE) and AdCMVluc (MOI=1). Forty eight hours after transduction, the amount of virus present in the lysate of cells was measured by a plaque assay in 293 cells.

Figure 11 shows enhancement of E1-defective adenoviral transgene expression by pE1FR administration. Nude mice engrafted with human lung adenocarcinoma tumors (A549 cell line) received an intratumoral injection of E1-defective virus AdCMVluc (10^8 pfu per 8-10 mm diameter tumor) mixed with plasmid pE1FR or pUC13 (3 μ g). One week later, luciferase expression in tumors was measured. Each bar represents one mouse with a pair of tumors, one treated with AdCMVluc and pE1FR and the other one with AdCMVluc and pUC13. The ratio of luciferase expression in the tumor treated with pE1FR versus the one treated with pUC13 is shown.

Figure 12 shows the E1A-like activity of IL-6 can be exploited to produce Ad312 virions in HepG2 cells and in a variety of

cell lines responsible to IL-6. Cells (1 to 4×10^5) were infected with wild type adenovirus or Ad5dl312 at an MOI of 10 in the absence or presence of 100 units/ml of rhIL-6. Six days later, cell were lysed and the amount of virus in the lysates was quantitated by plaque assay in 293 cells. For each cell line, bar from left to right represent wild type, wild type + IL-6, dl312, and dl312 + IL-6.

Figure 13 shows replication of Ad5dl312 and oncolytic effect in tumor cells without IL-6 addition. Ovarian carcinoma cells (OVCAR-3) were infected with E1-a deleted AD5dl312, wild type or E4-deleted Ad5dl1014 (MOI=10). **Figure 13A** shows that six days post-infection, cells were lysed and the amount of virus in the lysates was measured by plaque assay in 293 cells (for WT and dl312) or W162 cells (for dl1014). **Figure 13B** shows that in a separate experiment, seven days post-infection cells were fixed with formaldehyde and stained with crystal violet. No viable cells were found in wells with cells infected with WT and dl312 viruses in contrast to mock-infected and dl1014-infected cells.

Figure 14 shows that E1a-deleted virus dl312 can lyse human ovarian cancer cells. SW626 cells and two primary cultures of two ovarian tumors were infected with E1-a deleted Ad5dl312, wild type or E4-deleted Ad5dl1014 (MOI=10). Seven days post-

infection, cells were fixed with formaldehyde and stained with crystal violet. No viable cells were found after infection with the wild type and dl312 viruses in contrast to mock-infected and dl1014-infections.

5 **Figure 15** shows that normal peritoneal lining cells do not support the replication of the E1a-deleted Ad5dl312 adenovirus even in the presence of exogenous IL-6. Human mesothelial were cells isolated from normal peritoneal lining by mechanical disruption and collagenase D treatment. Cells were infected with E1-a deleted
10 Ad5dl312 or wild type control (MOI=10) in the absence or presence of IL-6. Twelve days post-infection cells were fixed with formaldehyde and stained with crystal violet. Cells remained viable when infected with Ad5dl312.

15

DETAILED DESCRIPTION OF THE INVENTION

The present invention addresses the two major limitations of replicative adenoviral agents (viruses and vectors) in
20 their application to cancer gene therapy: the efficacy of transduction and the specificity of replication. Adenovirus binds to the

coxsackievirus-adenovirus receptor, CAR, in the cellular membrane using the C-terminal globular domain of the viral fiber, the knob [41]. Since a limited amount of coxsackievirus-adenovirus receptor is present in tumors, one means to enhance infectivity would be to provide additional binding pathways [20,21]. Therefore, two methods have been developed to modify adenovirus binding. The first method uses a Fab fragment of an anti-knob antibody conjugated to a ligand of a cellular receptor, while the second method comprises direct genetic modification of the knob sequence.

One important advantage of direct genetic modification is that the progeny will carry the modified fiber, thereby retaining the replicative virus' enhanced infectivity trait through the amplification cycles. Wickman et al. have generated adenoviruses with chimeric fibers in which the ligand is connected to the carboxy terminal position of the fiber [26]. This carboxy terminal location is not always appropriate because the addition of more than 20-30 heterologous amino acid residues can result in the loss of fiber trimerization and binding to the capsid. Furthermore, the three-dimensional structure of the fiber indicates that the carboxy terminal end points towards the virion, and therefore, away from the cell surface [42]. For these reasons, the HI loop was used herein as an

exposed and amenable site for the incorporation of exogenous sequences.

With regard to the efforts to increase the specificity at the level of virus replication, methods have been developed to confer regulated-replication or conditional-replication competency to adenoviral vectors based upon complementing, in *trans*, the essential early genes that are missing in the replication-defective vectors. In this way, E1-deleted and E4-deleted vectors have been transcomplemented by conjugating them to E1 or E4 expression plasmids [43,44]. This method enables the vectors to replicate, thereby augmenting their transduction ability. Methods have also been explored that allow the continuous replication of the vector, such as using the E1a-like activity provided by interleukin 6 to enable replication of E1a-deleted vectors.

It has been recognized that the major limitation in several strategies of cancer gene therapy resides in the need to transduce the majority of cells of a tumor. With the exception of a limited bystander effect described in some strategies, the cells that are left untransduced will jeopardize and reduce any therapeutic effect. Adenoviral vectors are limited in this regard by the paucity of its receptor, CAR, in tumors [20-23]. It is a goal of the present

invention to improve the infectivity of adenoviral vectors by providing additional pathways to cell binding besides CAR. Previous data has shown that modification of the HI loop of the fiber is a feasible strategy to add new ligand motifs into the fiber. An RGD motif has already been incorporated into the fiber of regular E1-deleted vectors and been shown to enhance the therapeutic effects *in vivo*.

The present invention describes the incorporation of the RGD-modified fiber into replicative adenoviral vectors. The present invention further describes methods to enhance the specificity of the replication of these replicative adenoviral vectors. The current methods of mutating E1, or regulation of E1 with tumor-specific promoters, are both very rational approaches, but may prove not selective enough for several reasons. In the case of E1 deletions, the main limitation lies in incomplete knowledge of the role of these proteins in the viral replicative cycle and in controlling the cell cycle. For example, adenovirus may use a p53-dependent mechanism to release the progeny from the infected cell [38]. This would predicate a positive role for p53 in virus production and would reduce the yields of virus in p53-deficient cells. On the other hand, other viral proteins besides E1-55K may block p53 function, such as E4, and this

would allow the 55K- to replicate in p53+ cells [37]. In any case the specificity of a 55K- for p53-defective cells is controversial [35,36]. Regarding to strategies based on regulation of E1 it is a concern that promoters can lose certain degree of specificity when inserted into the viral genome [39]. The presence of E1-like activity in uninfected cells could also pose a problem for the specificity achieved with both vectors. In this regard, some replication of E1 vectors has been observed in many different cell lines [40].

Therefore, it is desirable to improve the replication selectivity of replicative adenoviral vectors for tumors by achieving tumor-selective regulation of key early genes other than E1, such as E2 or E4. An adenovirus-polylysine-DNA transcomplementation system has been developed as a means to evaluate replication. This replication-enabling system is used to analyze the efficacy and specificity of tumor-specific replication mechanisms based on the regulation of the E4 or E2 genes. In the transcomplementation system, plasmids encoding E2 or E4 under the control of different tumor-specific promoters are used to screen for mechanisms that confer selective replication. Ultimately, selective replication will involve the incorporation of the regulated E4 or E2 into the viral genome to achieve continuous replication. Accordingly, after the

tumor-selective replication has been demonstrated, these regulatory mechanisms are incorporated into a single viral vector. Optimally, these regulatory mechanisms are combined with the fiber modification described above to enhance infectivity.

5 Initial tumor models are based on cell lines with differential expression of the PSA protein: LNCaP and DU145. Tumors derived from lung adenocarcinoma cell lines and ovarian cell lines are used to evaluate viruses with promoters such as Carcinoembryonic antigen (CEA) or secretory leukoprotease inhibitor
10 (SLPI). Therapeutic effects are only observed in tumors derived from the cell lines that allow the expression of the tumor-specific controlled E4 or E2, that is, replication of the virus. In these permissive cell lines, higher therapeutic advantage is observed for the RGD-modified virus relative to the unmodified virus.

15 The present invention is directed towards an infectivity-enhanced conditionally-replicative adenovirus. This adenovirus possesses enhanced infectivity towards a specific cell type, which is accomplished by a modification to an HI loop of a fiber knob from a wildtype adenovirus and results in enhanced infectivity relative to
20 the wildtype adenovirus. The adenovirus also has at least one

conditionally regulated early gene, such that replication of the adenovirus is limited to the specific cell type.

Preferably, the cell is a tumor cell, and generally, modification to the HI loop results in CAR-independent gene transfer.

5 A preferred modification to the HI loop comprises introducing a ligand into the HI loop, and representative ligands include physiological ligands, anti-receptor antibodies and cell-specific peptides. Additionally, the ligand may comprise a tripeptide having the sequence Arg-Gly-Asp (RGD), or more specifically, a peptide
10 having the sequence CDCRGDCFC.

The early gene may be conditionally regulated by means consisting of a tissue-specific promoter operably linked to an early gene (*e.g.*, E1, E2 and/or E4) and a mutation in an early gene (*e.g.*, E1, E2 and/or E4). Representative tissue-specific promoters are the
15 prostate specific antigen (PSA), Carcinoembryonic antigen (CEA), secretory leukoprotease inhibitor (SLPI), and alpha-fetoprotein (AFP).

Additionally, the adenovirus may carry a therapeutic gene in its genome. In conjunction with the above-mentioned
20 therapeutic gene, a method of providing gene therapy to an individual in need of such treatment is disclosed herein, comprising

the steps of: administering to the individual an effective amount of an infectivity-enhanced conditionally-replicative adenovirus. When the therapeutic gene carried by the adenovirus is, for instance, a herpes simplex virus thymidine kinase gene, the present invention
5 further provides for a method of killing tumor cells in an individual in need of such treatment, comprising the steps of: pretreating the individual with an effective amount of an infectivity-enhanced conditionally-replicative adenovirus expressing the TK gene; and administering ganciclovir to the individual. Generally, the individual
10 has cancer.

The present invention is also directed towards a method of infectivity-enhanced and conditionally-replicative adenoviral gene therapy in an individual in need of such treatment, comprising the steps of: administering to the individual a therapeutic dose of an
15 infectivity-enhanced conditionally-replicative adenovirus. Representative routes of administration are intravenously, intraperitoneally, systemically, orally and intratumorally. Generally, the individual has cancer and the cell is a tumor cell.

In accordance with the present invention, there may be
20 employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such

techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic
5 Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription and Translation" [B.D. Hames & S.J. Higgins eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984). Therefore, if appearing
10 herein, the following terms shall have the definitions set out below.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule,
15 and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (*e.g.*, restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure herein according to the normal convention of giving only the sequence in the 5' to 3'
20 direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA).

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. A "replicon" is any genetic element (*e.g.*, plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; *i.e.*, capable of replication under its own control. An "origin of replication" refers to those DNA sequences that participate in DNA synthesis. An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is "operably linked" and "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

In general, expression vectors containing promoter sequences which facilitate the efficient transcription and translation of the inserted DNA fragment are used in connection with the host. The expression vector typically contains an origin of replication, promoter(s), terminator(s), as well as specific genes which are capable of providing phenotypic selection in transformed cells. The transformed hosts can be fermented and cultured according to means known in the art to achieve optimal cell growth.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence. A "cDNA" is defined as copy-DNA or complementary-DNA, and is a product of a reverse transcription reaction from an mRNA transcript. An "exon" is an expressed sequence transcribed from the gene locus, whereas an "intron" is a non-expressed sequence that is from the gene locus.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell. A "*cis*-element" is a nucleotide sequence, also termed a "consensus sequence" or "motif", that interacts with other proteins which can upregulate or

downregulate expression of a specific gene locus. A "signal sequence" can also be included with the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell and directs the polypeptide to the appropriate cellular location. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

The term "oligonucleotide" is defined as a molecule comprised of two or more deoxyribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide. The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, *i.e.*, in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

Primers are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need
5 not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer,
10 provided that the primer sequence has sufficient complementarity with the sequence or hybridize therewith and thereby form the template for the synthesis of the extension product.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to enzymes which cut double-stranded
15 DNA at or near a specific nucleotide sequence.

"Recombinant DNA technology" refers to techniques for uniting two heterologous DNA molecules, usually as a result of *in vitro* ligation of DNAs from different organisms. Recombinant DNA molecules are commonly produced by experiments in genetic
20 engineering. Synonymous terms include "gene splicing", "molecular

cloning" and "genetic engineering". The product of these manipulations results in a "recombinant" or "recombinant molecule".

A cell has been "transformed" or "transfected" with exogenous or heterologous DNA when such DNA has been introduced
5 inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a vector or plasmid. With respect to eukaryotic cells, a stably transformed cell is one in
10 which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A
15 "clone" is a population of cells derived from a single cell or ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations. An organism, such as a plant or animal, that has been transformed with exogenous DNA is termed "transgenic".

20 As used herein, the term "host" is meant to include not only prokaryotes but also eukaryotes such as yeast, plant and animal

cells. A recombinant DNA molecule or gene can be used to transform a host using any of the techniques commonly known to those of ordinary skill in the art. Prokaryotic hosts may include *E. coli*, *S. typhimurium*, *Serratia marcescens* and *Bacillus subtilis*.

5 Eukaryotic hosts include yeasts such as *Pichia pastoris*, mammalian cells and insect cells, and more preferentially, plant cells, such as *Arabidopsis thaliana* and *Tobaccum nicotiana*.

A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not
10 found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, the coding sequence is a construct where the coding
15 sequence itself is not found in nature (*e.g.*, a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

20 As used herein, the terms "conditionally regulated" and "conditionally-replicative" refer to the expression of a viral gene or

the replication of a virus or a vector, wherein the expression of replication is dependent (*i.e.*, conditional) upon the presence or absence of specific factors in the target cell.

As used herein, the term "early genes" refers to those
5 adenoviral genes expressed prior to the onset of adenoviral DNA replication.

As used herein, the term "CAR-independent infectivity" refers to the entry of adenovirus into a cell by receptors different from the coxackie-adenovirus receptor (CAR).

10 As used herein, the term "RGD-integrin interaction" refers to the arginine-glycine-aspartic acid (RGD) residues in a peptide binding to the integrin receptor molecules.

As used herein, the term "replication-competent adenoviruses" refers to an adenovirus capable of replication (*i.e.*, an
15 adenovirus that yields progeny).

It is specifically contemplated that pharmaceutical compositions may be prepared using the novel adenovirus of the present invention. In such a case, the pharmaceutical composition comprises the novel adenovirus of the present invention and a
20 pharmaceutically acceptable carrier. A person having ordinary skill in this art would readily be able to determine, without undue

experimentation, the appropriate dosages and routes of administration of this adenovirus of the present invention. When used *in vivo* for therapy, the adenovirus of the present invention is administered to the patient or an animal in therapeutically effective amounts, *i.e.*, amounts that eliminate or reduce the tumor burden. It may be administered parenterally, preferably intravenously, but other routes of administration will be used as appropriate. The dose and dosage regimen will depend upon the nature of the cancer (primary or metastatic) and its population, the characteristics of the particular immunotoxin, *e.g.*, its therapeutic index, the patient, the patient's history and other factors. The amount of adenovirus administered will typically be in the range of about 10^{10} to about 10^{11} viral particles per patient. The schedule will be continued to optimize effectiveness while balanced against negative effects of treatment. See Remington's Pharmaceutical Science, 17th Ed. (1990) Mark Publishing Co., Easton, Penn.; and *Goodman and Gilman's: The Pharmacological Basis of Therapeutics* 8th Ed (1990) Pergamon Press; which are incorporated herein by reference. For parenteral administration, the adenovirus will most typically be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle.

Such vehicles are preferably non-toxic and non-therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate may also be used.

5 Liposomes may be used as carriers. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, *e.g.*, buffers and preservatives. The immunotoxin will typically be formulated in such vehicles at concentrations readily reconizable to those having ordinary skill in this art.

10 The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion:

15

EXAMPLE 1

Enhanced tumor transduction with adenoviral vectors modified with a ligand attached to the fiber

As a first approach towards enhancing the infectivity of
20 adenoviral vectors and to demonstrate the tumor transduction advantage of vectors with altered tropism over unmodified vectors,

an anti-fiber antibody conjugated to fibroblast growth factor (FGF2) was used. The Fab portion of the anti-knob antibody, 1D6.14, which is capable of blocking the interaction of the fiber with its cognate cellular receptor, was chemically conjugated to FGF2. The resulting Fab-FGF2 conjugate was complexed with adenoviral vectors expressing luciferase or β -galactosidase reporter genes to compare the transduction efficiency of the modified and unmodified vectors. Vector modification increased the level of gene expression more than 9-fold, as measured by luciferase activity (Figure 1A), largely due to transduction of a greater percentage of target cells as seen by β -galactosidase staining (Figure 1B). This experiment clearly demonstrates that a retargeted adenoviral vector can overcome the inefficacious transduction observed in certain cell lines transduced poorly by adenoviral vectors.

To compare the therapeutic effect of an FGF2-modified vector to an unmodified vector in established tumors, the conjugate was then mixed with an adenovirus expressing HSV-TK (AdCMVHSV-TK). Treatment with the modified vector of SKOV3 ovarian carcinomas established in nude mice followed by administration of the prodrug, ganciclovir, resulted in a significant prolongation of survival when compared with the unmodified vector plus ganciclovir

(Figure 2). Thus, retargeting can increase the *in vivo* therapeutic effect of adenoviral vectors against tumors. It is clear that the infectivity of tumors by unmodified adenovirus is not optimal and modification of the capsid to alter the tropism of the virus is a direct approach to increase this infectivity.

EXAMPLE 2

10 Genetic modification of the HI loop of the fiber provides enhanced infectivity to adenoviral vectors

The Fab-ligand conjugation method described in Example 1 only modifies the tropism of the vector prepared for inoculation. In the context of a replicative vector, it is advantageous to modify the tropism of the vector that replicates in the tumor as well. With this rationale, a genetic modification of the fiber is necessary for replicative vectors because it is carried over to the progeny. As a simple and potent strategy for retargeting, the sequence of the fiber was genetically modified. Based on the three-dimensional model of the fiber knob [42], targeting ligands were inserted into the HI loop of the fiber (Figure 3). This loop is flexible, exposed on the outside of

the knob, is not involved in fiber trimerization and its variable length in different Ad serotypes suggests that insertions or substitutions do not affect the fiber stability.

As a ligand to introduce into the HI loop of the fiber knob, the sequence coding for an RGD peptide, CDCRGDCFC, was chosen. This RGD sequence is known to target tumors by binding with high affinity to several types of integrins [45,46]. It was hypothesized that an adenoviral vector able to bind via fiber-RGD/integrin interaction would not depend upon the presence of the CAR receptor in tumors to be effective, and would therefore target tumors more efficiently than the unmodified vector counterpart.

The DNA sequence encoding the peptide was cloned into the *EcoRV* site of the knob domain in a plasmid containing the fiber sequence. The wild type fiber of an E1,E3-deleted adenoviral vector expressing the luciferase gene, AdCMVLuc, was replaced with the RGD-modified fiber by homologous recombination in bacteria [47]. After homologous recombination, the genome of the new adenoviral vector was released from the plasmid backbone by digestion with *PacI*. To use the firefly luciferase gene, the internal *PacI* site of this gene was eliminated by introducing a silent mutation. The plasmid obtained as a result of these DNA recombinations was then utilized

for transfection of 293 cells to rescue Ad5lucRGD. The presence of RGD in the virus was confirmed by PCR as well as by cycle sequencing of viral DNA isolated from CsCl-purified virions of Ad5lucRGD.

5 To demonstrate that the genetic modification of the fiber was able to confer CAR-independent infectivity to the modified vector, the unmodified AdCMVLuc and the modified Ad5lucRGD vectors were used to transduce 293, HUVEC, and RD cell lines, which express high, moderate, and low levels of CAR, respectively. The
10 CAR-independent infection was further analyzed using competitive inhibition by recombinant Ad5 fiber knob protein, known to efficiently block virus binding to CAR receptor. Luciferase expression in 293 cells mediated by the unmodified virus, AdCMVLuc, was efficiently blocked by recombinant knob protein
15 (Figure 4A). Depending on the multiplicity of infection (MOI) used, knob protein blocked 85% to 93% of luciferase activity in AdCMVLuc-transduced cells. In contrast, the same concentration of knob was able to block only 40% to 60% of Ad5lucRGD-mediated gene expression in 293 cells, indicating that in addition to the fiber-CAR
20 interaction utilized by the wild type Ad5, Ad5lucRGD is capable of using an alternative, CAR-independent, cell entry pathway. Of note,

the contribution of that alternative mechanism of cell binding was quite significant, providing 40% to 60% of overall gene transfer to 293 cells. Luciferase expression in HUVEC cells transduced with Ad5lucRGD was about 30-fold higher than with AdCMVLuc (Figure 4B). The effect of Ad5 fiber knob on AdCMVLuc-mediated transduction was less dramatic than in 293 cells, consistent with a relative lack of CAR in the HUVEC. Most importantly, recombinant knob protein did not inhibit the levels of luciferase expression directed by Ad5lucRGD. The luciferase activity detected in RD cells transduced with AdCMVLuc was extremely low: at an MOI of one pfu/cell, it was almost equal to the background level of mock-infected cells (Figure 4C). In contrast, the level of transgene expression achieved with Ad5lucRGD was 16- to 47-fold higher than with AdCMVLuc, and expression was not inhibited by the fiber knob.

These experiments clearly showed that incorporation of the RGD peptide into the fiber of Ad5lucRGD resulted in dramatic changes in virus-to-cell interaction by providing an alternative CAR-independent cell attachment pathway. Of note, the insertion of the RGD sequence in the HI loop did not abrogate the CAR-mediated entry pathway, so the modified vector has a two independent mechanism to bind to the cells. As the present invention shows, this

contributes to the enhanced infectivity of the modified vector in all cell lines and tumors tested.

5

EXAMPLE 3

Enhanced tumor transduction via RDG-fiber modification

To determine if the RGD sequence incorporated into the HI loop of the fiber could increase the infectivity of tumors, the ability of the modified vector to deliver genes to cultured human ovarian cancer cells was examined. Characterization of two cell lines, SKOV3.ip1 and OV-4, by flow cytometry showed that they both express moderate-to-high levels of $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins. SKOV3.ip1 also expresses a high level of CAR, whereas OV-4 only modestly expresses CAR.

The incorporation of recombinant RGD-containing fiber protein in the Ad5lucRGD vector dramatically improved the ability of the virus to efficiently transduce these cells (Figure 5A). At different MOIs tested, Ad5lucRGD-transduced cultures of SKOV3.ip1 cells showed 30-fold to 60-fold increase in luciferase activity compared to cells transduced with control virus. Interestingly, while the purified

fiber knob blocked over 90% of AdCMVLuc-mediated gene transfer, it could only block 20% of luciferase activity in Ad5lucRGD-treated cells, indicating a majority of CAR-independent entry mechanisms for Ad5lucRGD. In OV-4 cells, the transduction efficiency achieved with the RGD-modified vector was 300- to 600-fold higher than the unmodified one (Figure 5B). Again, when the fiber knob was used as an inhibitor of CAR-mediated cell entry, it did not have any significant effect on Ad5lucRGD-mediated gene delivery, strongly suggesting that this virus primarily utilizes RGD-integrin interaction to bind to target cells.

The utility of the Ad5lucRGD vector was next evaluated in the context of primary tumor cells. In this regard, recent human clinical trials have pointed out the disparity between the efficacy of adenoviral vectors in various model systems and in the clinical context, where rather low transduction efficiencies have been noted [20-23]. As integrins have been shown to be frequently overexpressed by various epithelial tumors, vector targeting to these cell surface receptors provides a means to achieve CAR-independent gene transfer [46].

In the experiments described herein, ovarian cancer cells obtained from two patients was treated with either Ad5lucRGD or

AdCMVLuc in the presence or absence of blocking knob protein. Luciferase expression in cells treated with AdCMVLuc was extremely low (Figure 6), thereby indicating inability of adenoviral vectors containing unmodified fibers to efficiently infect ovarian cancer cells.

5 Strong inhibition by the fiber knob on AdCMVLuc-mediated luciferase expression suggests that the fiber-CAR interaction is the only pathway this virus can use to infect this type of cell. In contrast, Ad5lucRGD directed levels of transgene expression two- to three-orders of magnitude higher than those detected in AdCMVLuc-
10 transduced cells. The knob blocked 20% of the gene transfer at an MOI of 1 pfu/cell, and no effect was observed at an MOI of 10 pfu/cell.

The observations of enhanced infectivity have been extended to other tumor cell types besides ovarian carcinoma. In six
15 human non-small cell lung adenocarcinoma cell lines, one human mesothelioma cell line, and one rat mesothelioma cell line, the luciferase expression level achieved with the RGD-modified vectors was always higher than the level achieved with the non-modified vector at a variety of different MOIs (Figure 7).

20 The increased efficacy of infection of the RGD-modified vector was also measured in time course experiments in which the

incubation time of the virus with the cells was limited. The transduction efficiency was always better with the modified vector and the differences were more marked at shorter times of infection: the RGD-modified vector produced a 1000-fold greater luciferase
5 expression when only 7 minutes of adsorption were allowed (Figure 8). At longer adsorption times, the differences between the modified and non-modified vectors were reduced to 10-fold. This difference could have important implications in adenoviral-mediated gene therapy because the time of exposure of the vector to the tumor
10 target cells is expected to be limited by the intratumoral high pressure.

Overall, this data points out the importance of providing an alternative entry pathway to adenoviral vectors for the infectivity of tumors. In all cell lines and tumor types analyzed, a vector that
15 can use the natural entry pathway via primary binding to CAR and an additional entry pathway via binding to integrins transduces more efficiently than a vector that only can use the natural CAR receptor.

EXAMPLE 4

Replication-competent, E1-transcomplementation vectors

Most replication-defective adenovirus vectors in
5 preclinical and clinical use have deleted E1A and E1B genes [14].
These deletions render the vector unable to replicate, or replication-
incompetent, and these vectors can replicate only when E1 proteins
are supplied in *trans*. These replication-incompetent vectors
transduce the cells that they infect but they do not produce any
10 progeny.

A conditional replication enablement system for
adenovirus has been developed in which the E1 genes are supplied in
trans to cells infected with E1-deleted vectors [31,48,49] (Figure 9).
The replication-enabling system has been developed primarily as a
15 means of amplifying transduction in tumor nodules. In order to
achieve a more extensive amplification of the vector and lysis of
tumor cells, the secondarily produced vector should propagate
continuously in tumor cells. Replication-enabling has been achieved
by linking plasmids encoding the E1 proteins to the exterior of the
20 capsid [31,44,48] or separately introducing the plasmid using cationic
lipids [49]. These experiments provided evidence that replication-

enabling systems could achieve amplification of the *in vivo* therapeutic response of an adenoviral vector carrying HSV-TK [49]. E4-deleted adenoviruses have also been transcomplemented with a plasmid containing the E4 open reading frame 6 gene or the complete
5 E4 region [44]. E4 transcomplementation is important in the context of reducing immunogenicity and increasing long-term gene transfer [14].

In order to further enhance the utility of the replication-enabling system, it is a goal of the present invention to reduce the
10 possibilities of recombination between the E1-deleted vector and the transcomplementing plasmid. This recombination would generate replication-competent adenoviruses (RCA). Therefore, an E1 expressing plasmid has been constructed, pE1FR, in which E1a and E1b sequences are in tandem but oriented in opposite 5' to 3'
15 direction. Cells co-transduced with this plasmid and an E1-defective adenoviral vector using cationic liposomes resulted in replication-defective adenovirus production levels comparable to that achieved by co-transduction of the virus and pE1 (Figure 10) [49]. Comparable results have been obtained with HeLa, A549 and SKOV3-ip1 cell
20 lines.

This demonstrates that pE1FR can transcomplement E1-deleted vectors and convert the infected cells into vector-producing cells. To demonstrate that this vector could also enhance the tumor transduction achieved with an E1-deleted vector *in vivo*, tumors
5 were injected with E1-defective virus mixed with pE1FR, or a plasmid control. Assessment of the luciferase content showed that 6 out of 8 tumors had increased luciferase activity in the pE1FR group relative to the controls (Figure 11).

This data indicates that E1-expression vectors, such as
10 pE1FR, represent a feasible way to increase the *in vivo* transduction efficiency of E1-deleted vectors in tumors. The amplification of the transduction efficiency achieved with a system such as the replication-enabling system is limited, however, by the inability of the vector progeny to keep replicating. The replication-enabling
15 function needs to be carried over in the vectors produced by the tumor cells to allow repeated cycles of replication.

EXAMPLE 5

Replication competent vectors dependent upon IL-6

As shown in the data above, the replication-enabling
5 system has been developed primarily as a means of amplifying
transduction in tumor nodules. Methods have also been explored to
achieve a more extensive amplification of the vector and subsequent
lysis of tumor cells. To fulfill this goal, the secondarily produced
vector should propagate continuously in tumor cells and incorporate
10 a regulatory mechanism that confines this propagation to the tumor.
Ela 12s and 13s adenoviral proteins are necessary to induce the
expression of other viral genes, and therefore, an Ela-deleted vector
is impaired in its replication [14]. It has been reported that
interleukin 6 can induce transcription factors that are able to
15 substitute for the Ela activity of adenovirus [50].

To explore whether an Ela-deleted vector such as
Ad5dl312 could replicate in the presence of IL-6 in different cancer
cell lines, cells were infected with dl312 in the presence of IL-6 and
the progeny were examined (Figure 12). In all cell lines, infectious
20 virions were produced to a certain extent in the presence and
absence of IL-6, although in lower amounts than the wild type

adenovirus. The effects of IL-6 in dl312 production were markedly seen in two cell lines: HepG2 and EJ. In HepG2 cells, IL-6 resulted in a 1.5 log increase of viral production.

These experiments demonstrate that the IL-6-inducible
5 E1a-like activity can complement the E1a deletion during infection of HepG2 and EJ cells. To overcome the requirement of exogenous IL-6, carcinomas, *e.g.*, cervical, chorio, and ovarian, that have an IL-6 autocrine loop [51-53] were infected with the E1A-deleted virus, dl312. OVCAR-3 and SW626 cells have a functional IL-6 autocrine
10 loop [53]. Upon infection of OVCAR-3 cells with Ad5dl312, or wild type or E4-deleted control viruses, Ad5dl312 was produced to levels similar to levels produced by the wild type control, even in the absence of IL-6 (Figure 13). This IL-6-independent replication of E1a-deleted virus was also demonstrated in SW626 cells and
15 primary cultures of ovarian tumors (Figure 13).

These results indicate that cells with an autocrine loop of IL-6 can selectively support the replication of Ad5dl312 without the addition of exogenous IL-6, and that these cells are lysed by the E1a-deleted virus. The effects of the E1a-deleted virus in normal cells
20 was examined. To test the ability of this virus to propagate in normal cells adjacent to ovarian tumors, human mesothelial cells

isolated from peritoneal lining tissue were infected. Contrary to the wildtype virus control, Ad5dl312 did not replicate in these cells even in the presence of IL-6 (Figure 15).

Overall, this data indicates that Ela-deleted adenovirus
5 can be complemented by the IL-6-induced Ela-like activity found in several tumors. Ela-deleted vectors are, however, limited by the fact that Ela intrinsic activity has been noted in normal cells [54]. IL-6 production, in the other hand, could result from the injection of the vector in an immunocompetent host and this natural inflammatory
10 response would result in nonspecific complementation. Clearly, new mechanisms of tumor-specificity need to be incorporated to control the replication of adenoviral vectors.

Summary

15 The clinical benefits of cancer gene therapy achieved with non-replicative adenoviral vectors have been hampered by the significant number of cells in a tumor which have been left unaffected by the direct or indirect effects of the transgenes. Conditional replicative adenoviruses may represent a significant
20 improvement to solve this problem. but efficient infectivity and

tumor-selective replication need to be achieved to realize their full potential.

The importance of the modification of the adenoviral capsid to increase the binding of the vector to the tumor cells has
5 been demonstrated herein. An integrin-binding RGD motif inserted in the HI loop of the adenoviral fiber confers an additional binding pathway besides the natural coxsackie-adenovirus receptor, and this dramatically increases the infectivity of the vector. The data herein also indicates that transduction efficiency can also be enhanced if the
10 vector is able to replicate in the tumor. A transcomplementation system has been developed as a means to evaluate the effects of replication on the transduction efficiency. This replication-enabling system also provides the opportunity to analyze the efficacy and specificity of different tumor-specific replication mechanisms before
15 incorporating these mechanisms into a single viral vector in a *cis*-complementation strategy that will allow continuous replication. In this regard, continuous tumor-selective replication has been shown using Ela-deletion mutants that propagate in tumors due to an Ela-like activity.

EXAMPLE 6

Incorporation of RGD-fiber into currently defined conditional replicative mutant viruses

5 As an initial approach towards comparing the therapeutic potential of an RGD-modified versus an unmodified replicative adenovirus, conditional replicative mutants that have been previously described were chosen. Deletion of the E1b-55K protein was designed to confer selective replication to adenoviruses in cells
10 lacking functional p53 [30]. In a similar way, deletion of the Rb-binding sites of E1a has been proposed to achieve selective replication in cells lacking Rb. These deletion mutants are used as established models of selective replication-competent viruses.

 The initial plasmid to construct these deletions is pXC1,
15 which contains adenoviral sequences from basepair 22 to 5790 (Microbix, Hamilton, Canada). For the E1b55K deletion, the region from *Sau3A1* (Ad5#2426) to *BglII* (Ad5#3328) is removed by ligation of the 1 kb *XbaI-Sau3A1* DNA fragment with the 7.9 kb *XbaI-BglII* DNA fragment to yield plasmid pXC-55K-. For an E1a
20 deletion construct that abrogates binding to Rb, a derivative of pXC1 (pXC1d24) is obtained with E1a deleted in residues 121 to 128 (Dr.

Juan Fueyo, MDACC). This deletion affects the residues of the conserved region 1 of E1a necessary to bind Rb [55]. These E1b and E1a deletions are incorporated into the viral genome by homologous recombination with plasmid pVK503, containing either an unmodified fiber or an RGD-modified fiber. From the plasmids obtained by homologous recombination, the unmodified 55k- and d24 mutants are generated by releasing the viral genome with *PacI* and transfecting into 293 cells. Viruses are amplified and purified by double CsCl gradient, and titered in 293 cells for *in vitro* and *in vivo* experiments. The presence of mutated E1, altered fiber, and contaminating wild type E1, is analyzed by PCR as well as by sequencing of viral DNA isolated from CsCl-purified virions.

15

EXAMPLE 7

Evaluation of infectivity of RGD-modified conditional replicative viruses

Procedures described above are used to demonstrate that the RGD-modified 55K- and d24 virions bind to integrins. ELISAs are performed with immobilized virions incubated with purified $\alpha v\beta 3$

integrins and anti- α subunit monoclonal antibody, VNR139. The modified replicative viruses are examined to determine if they are able to bind cells via a CAR-independent pathway. 293, HUVEC, and RD cells are used, as enhanced RGD-mediated transduction of these cell lines has already been demonstrated. For binding analysis, virions are labeled with ^{125}I and incubated with cells. Recombinant knob protein is used as an inhibitor to measure CAR-independent binding. Infectivity of modified and unmodified 55K and d24 mutants in ovarian, lung and other tumor cell lines, as well as in primary tumors, are compared. These experiments indicate that the RGD-modified viruses infect tumor cells more efficiently than the non-modified vectors.

15 EXAMPLE 8

Evaluation of oncolytic potential of RGD-modified conditional replicative viruses

The effect of RGD modification on the efficiency of propagation and oncolysis was determined next. Ovarian and lung adenocarcinoma cell lines are infected with MOIs from 10^{-4} to 1

(infection in serial dilutions) and at different times post-infection (2 to 14 days) viral propagation is measured. C33A and Saos-2 are also included as positive control cell lines permissive for 55K and d24, respectively. Propagation is quantified in three ways: amount of viral DNA produced (measured by Southern blots with viral DNA as a probe), amount of virions produced (measured by plaque assays with 293 cells), and cell lysis (measured by staining living cells with crystal violet dye). In each cell line, more DNA replication, virus production, and cell lysis per input viral particle is observed with the modified mutants versus their unmodified counterparts due to enhanced infectivity in primary and secondary infections. These experiments indicate that the RGD modification increases the propagation efficacy of the 55K and d24 mutants in each of the cell lines. The effect of enhanced-infectivity mediated by the RGD modification in secondary infections needs to be studied further. To discriminate the effects of the enhanced infectivity achieved with the inoculated virus in the primary infection versus the effects of enhanced infectivity achieved with the virus produced in the tumor cells (secondary infections), tumor cell lines are infected using a high MOI to achieve complete infection and subsequently mixed with

increasing numbers of uninfected cells. The three parameters of propagation are then measured in time course experiments.

5

EXAMPLE 9

Evaluation of tumor-selective E2 and E4 functions

One goal of the present invention was to demonstrate that tumor-selective regulation of E4 and E2 can confer tumor-selective replication to adenovirus. It has previously been shown that E4-deleted adenoviruses can be transcomplemented by conjugating an E4 expression plasmid into their capsid [44]. In this regard, plasmids such as pCEP-ORF6, that contain the E4 ORF6 under a constitutive promoter, can be used to transcomplement E4 deleted viruses, such as dl1014. In order to achieve tumor-selective expression of E4-ORF6, tumor-specific promoters are substituted for the CMV promoter. Among several tumor or tissue selective promoters that have been used in restricting expression of genes to tumor cells, the promoter of the prostate specific antigen (PSA) is used initially. PSA is expressed in prostate cells and has been used to direct expression of TK to prostate tumors [56]. This promoter was chosen to control

20

E4 and E2 in the context of replicative adenoviruses because it has already been used to control E1 in this context (obtained from Dr. Chris Baigma [57]). The promoter is subcloned in front of the E4ORF6 in plasmid pCEP-ORF6 to obtain a pPSA-ORF6 expression plasmid. To
5 evaluate the conditional replicative phenotype of a PSA-ORF6-regulated virus, this plasmid is conjugated with the E4-deleted virus, dl1014. Conjugates with pCEP-ORF6 or irrelevant plasmids are used as positive and negative controls, respectively. These Ad5dl1014 adenovirus-polylysine-plasmid conjugates are used to infect tumor
10 cell lines that express prostate specific antigen, such as LNCaP, and cell lines that do not express prostate specific antigen, such as DU145 or PC3. In time course experiments, viral replication is measured at the DNA level by Southern blot. The amount of virus produced from the molecular conjugates is measured by plaque assays in W162 cells
15 [44]. dl1014 DNA replication and virus production is observed in all cell lines when using pCEP-ORF6, but only in the PSA-expressing cell line, LNCaP, when using pPSA-ORF6.

These results indicate whether the E4 can be used to control the replication of E4-deleted adenoviruses and whether the
20 PSA promoter restricts this replication to cells expressing PSA. As a reference background and for comparison purposes, a PSA-E1

plasmid is constructed as a derivative of the E1 constructs used in the replication-enabling system, such as pE1FR. An E1-deleted vector and 293 cells are used to evaluate the selective replication conditional to the expression of prostate specific antigen. The differential propagation and the levels of virus production obtained with PSA-E4 and PSA-E1 regulation indicates which of these regulatory mechanism renders better selectivity of replication when used independently.

A similar strategy is followed to achieve selective expression of E2. E2-expression plasmids transcomplement E2-defective viruses using the replication-enabling system. The function of the three open reading frames of E2 (DNA binding protein, terminal protein, and polymerase) are subcloned into separate plasmids. These open reading frames of E2 are then placed under the regulation of the PSA promoter. Appropriate E2-defective mutant viruses, such as Ad5ts125 which contains a temperature-sensitive mutation of E2-DBP, are used to construct the corresponding adenovirus-polylysine-DNA conjugates. As above, these conjugates are used to infect LNCaP, DU145 and PC3 cell lines. Viral DNA replication is measured by Southern blot. Cell lines

expressing E2 are used to measure the amount of E2-deleted viruses produced by plaque assays [58].

5

EXAMPLE 10

Construction of RGD-fiber adenoviruses with tumor-selective E4 or E2 transcriptional units

It is a goal of the present invention to combine the fiber
10 modification with the replication-regulatory mechanisms. Towards
this direction, the E4 and/or E2 construct(s) that demonstrated
conditional regulation in the replication-enabling system replace the
endogenous viral E4 and/or E2 transcriptional unit. For this, the
region that is to be modified is subcloned into a small plasmid to
15 facilitate its manipulation. This region is then removed from the
plasmid and co-transformed into competent bacteria with a plasmid
containing the complete viral genome. The recombination between
the viral sequences flanking the modified region and the homologous
sequences in the larger plasmid results in the incorporation of the
20 modified region into the adenoviral genome. Before the co-
transformation step, it is necessary to cut the large plasmid in a

unique site located in the middle of the homology region to avoid the presence of colonies derived from the large plasmid. As there are no available unique sites in the E4 or E2 promoter region, the RecA-assisted cleavage method will be used to restrict in the proper site
5 [59].

This method involves three steps: first, an oligonucleotide spanning the site to be cut in the E2 or E4 promoter region is annealed to the large plasmid in the presence of RecA protein (New England Biolabs, Beverly, MA) to form a three-stranded segment.
10 Second, a methylase recognizing this site is then used to methylate all the sites in the large plasmid except the one protected by the oligonucleotide. Finally, the oligonucleotide is removed by heat and the corresponding restriction endonuclease is used to cut the unique non-methylated site. Common site-specific methylases, such as *AluI*,
15 *HaeIII*, *HhaI*, *HpaII*, etc. and the corresponding restriction endonucleases are purchased from New England Biolabs. Plasmids containing the wild type fiber and plasmids with the modified RGD fiber are used. After the homologous recombination step, the larger plasmids containing the viral genomes with the substituted E4 or E2
20 regions are cut with *PacI* to release the viral genome. Finally, the viruses are obtained by transfection into E4 or E2 complementing cell

lines. Viruses are amplified and purified by double CsCl gradient, and titered in these cell lines for *in vitro* and *in vivo* experiments. The presence of the E4 or E2 transcription unit regulated with the tumor-specific promoter and of the mutated fiber is analyzed by PCR
5 as well as by sequencing of viral DNA isolated from CsCl-purified virions.

EXAMPLE 11

10

Localized models

Subcutaneous tumor nodules are established using the LNCaP and DU145 cell lines. Cells (10^7) are mixed 1:1 with Matrigel (Collaborative Bioproducts), loaded into syringes and injected
15 subcutaneously in a total volume of 200 μ l into the front flanks of athymic nude mice (2×10^6 cells per engraftment site). Initially, three pairs of viruses are compared: PSAE4-RGD versus PSAE4; PSA-E2 versus RGD-PSAE2; and PSA-E1 versus RGD-PSAE1. In a second phase, viruses with double E1/E4 or E1/E2 controlled transcriptional
20 units are also analyzed. Tumor nodules are injected with the appropriate adenovirus or vehicle control (PBS/10% glycerol) when

their volume (length x width $2 \times 1/2$) reaches 0.2 cm^3 . Injections are with a Hamilton syringe in a volume of $20 \text{ } \mu\text{l}$ ($1/10$ of tumor volume). The amount of virus injected per tumor is adjusted from 10^4 pfus (plaque forming units) to 10^8 pfus by serial dilution. A series of experiments are done to measure the tumor volume until regression or a maximum of 1 cm^3 . Another series of experiment are performed to measure the intratumoral amount of virus in a time course. This amount is measured by resecting the tumors and staining sections with anti-hexon antibody (Chemicon) and by extracting the virus from the tumors and measuring the viable virus in a plaque assay. In DU145 tumors, no therapeutic effect is observed with the PSA-controlled viruses. In LNCaP tumors, smaller tumors or complete tumor regressions is observed, and more intratumoral virus in tumors treated with the PSA-controlled replicative viruses is observed when compared to the non-replicative and vehicle control treated tumors. Smaller tumors or more frequent complete regressions are observed, likely due to higher amounts of intratumoral virus with the RGD-modified vector. These results demonstrate that the tumor-specific regulation of adenoviral genes, such as E4, allows replication *in vivo* in permissive tumors and also

demonstrates the therapeutic advantage of the RGD modification for a replicative adenovirus.

5

EXAMPLE 12

Local-regional and disseminated models

A murine model for ovarian cancer and liver metastases of colon cancer has been developed. These models have been useful
10 in demonstrating the utility of the RGD modification for non-replicative adenoviral vectors, and therefore, are used herein in the context of replicative adenoviruses containing tumor-specific promoters. The ovarian cancer model is a local-regional model that uses the human ovarian cancer cell line, SKOV3.ip1. As these cells
15 express SLPI, this model is useful to evaluate viruses in which the E4 or E2 gene is regulated by the SLPI promoter. This cell line has been serially passaged in SCID mice and selected for its ability to grow aggressively in the peritoneum [62]. Female SCID mice receive an i.p. injection of 2×10^7 cells in 0.5 ml of serum-free medium. Five days
20 after injection, tumors start to form at the peritoneum surface and the progression of the disease mimics the human disease. One week

after injection, the viruses (RGD-modified or the unmodified control) will be injected i.p. in a volume of 100 μ l. The therapeutic viruses are also intravenously injectioned. Virus dosages range from 10^4 pfus to 10^8 pfus. The therapeutic effect is measured by surviving
5 cells. The amount of replicating virus is measured in peritoneal lavages in time course experiments.

The model of colon cancer liver metastases uses LS174T human colon cancer cells and allows for expression of genes under the CEA promoter. In a surgical operation, cells (5×10^8) are injected
10 along the long axis of the spleen. Five minutes after the injection, the splenic vessels are tied off and the spleen is cut and removed. After the abdominal wall and skin are sutured, extensive liver metastases form in 7-10 days. Tail vein injection of RGD-modified and unmodified replicative adenoviruses to demonstrate systemic
15 treatment using this model. Liver metastases are counted in a time course experiment after virus injection.

These experiments provide *in vivo* data demonstrating selective replication and oncolytic potency of replicative vectors with restricted replication and enhanced infectivity. The RGD modification
20 in the fiber of replicative adenoviruses, along with tumor-selective

expression of E4 or E2 in addition to E1, increases the virus' propagation efficacy and ultimately its therapeutic efficacy.

EXAMPLE 13

5

Vertebrate Animals

Mice containing human tumors are used to evaluate the therapeutic potential of adenoviruses with enhanced infectivity and tumor-selective replication. Three types of models are used:
10 subcutaneous engrafted cell lines (prostate LNCaP and DU145), diffuse intraperitoneal engraftments (ovarian SKOV3-ip1), and liver metastases (colorectal carcinoma cell line LS174T). Adult (6-8 week old) athymic nu/nu mice are used in the subcutaneous and metastatic models and SCID mice are used in the intraperitoneal
15 model. Except for the prostate cell lines, female mice are used. Treatments include the RGD-modified, non-modified and vehicle control in a single injection for each dose. Intratumoral, intraperitoneal or intravenous administration of the viruses (according to the model used) is performed with unsedated mice
20 using gentle physical restraint. All mice are euthanized at the

conclusion of all experiments by CO₂ vapor sedation followed by Phenobarbital overdose.

The following references were cited herein:

1. Anderson, W.F., *Nature*, 1998. 392(6679 Suppl): p. 25-30.
- 5 2. Roth, et al., *J Natl Cancer Inst*, 1997. 89(1): p. 21-39.
3. Urban, J.L., et al., *J Immunol*, 1986. 137(9): p. 3036-41.
4. Paillard, F., *Hum Gene Ther*, 1998. 9(17): p. 2457-8.
5. Carbone, F.R., et al., *Immunol Today*, 1998. 19(8): p. 368-73.
6. Schreiber, H., *Tumor Immunology*, in *Fundamental*
10 *Immunology*, W.E. Paul, Editor. 1999, Lippincott-Raven Publishers:
Philadelphia. p. 1237-1270.
7. Cook, D.R., et al., *Cancer Biother*, 1994. 9(2): p. 131-41.
8. Moolten, F.L., *Cancer Gene Ther*, 1994. 1(4): p. 279-87.
9. Alemany, R., et al., *Cancer Gene Ther*, 1996. 3(5): p. 296-301.
- 15 10. Gomez-Manzano, C., et al., *Cancer Res*, 1996. 56(4): p. 694-9.
11. Rasmussen, et al., *Pharmacol Ther*, 1997. 75(1): p. 69-75.
12. Tanaka, T., et al., *Cancer Res*, 1998. 58(15): p. 3362-9.
13. Zhang, et al., *Cancer Metastasis Rev*, 1996. 15(3): p. 385-401.
14. Yeh, P. & M. Perricaudet, *FASEB J*, 1997. 11(8): p. 615-23.
- 20 15. Mittereder, N., et al., *J Virol*, 1996. 70(11): p. 7498-509.
16. Fallaux, F.J., et al., *Hum Gene Ther*, 1998. 9(13): p. 1909-17.

17. Smith, C.A., et al., J Virol, 1996. 70(10): p. 6733-40.
18. Deng, Y., et al., J Immunol, 1997. 158(4): p. 1507-15.
19. Worgall, S., et al., Hum Gene Ther, 1997. 8(1): p. 37-44.
20. Goldman, C.K., et al., Cancer Res, 1997. 57(8): p. 1447-51.
- 5 21. Miller, C.R., et al., Cancer Res, 1998. 58(24): p. 5738-48.
22. Hemmi, S., et al., Hum Gene Ther, 1998. 9(16): p. 2363-73.
23. Dmitriev, I., et al., J Virol, 1998. 72(12): p. 9706-13.
24. Krasnykh, V., et al., J Virol, 1998. 72(3): p. 1844-52.
25. Yoshida, Y., et al., Hum Gene Ther, 1998. 9(17): p. 2503-15.
- 10 26. Wickham, T.J., et al., J Virol, 1997. 71(11): p. 8221-9.
27. Miller, N. & J. Whelan, Hum Gene Ther, 1997. 8(7): p. 803-15.
28. Russell, S.J., Semin Cancer Biol, 1994. 5(6): p. 437-43.
29. Sinkovics, et al., Intervirology, 1993. 36(4): p. 193-214.
30. Kirn, & McCormick, Mol Med Today, 1996. 2(12): p. 519-27.
- 15 31. Goldsmith, K.T., et al., Hum Gene Ther, 1994. 5(11): p. 1341-8.
32. Kirn, D., et al., Nat Med, 1998. 4(12): p. 1341-2.
33. Rodriguez, R., et al., Cancer Res, 1997. 57(13): p. 2559-63.
34. Schuepbach, J. & C. Sauter, Cancer, 1981. 48(6): p. 1363-7.
35. Goodrum, F.D. & D.A. Ornelles, J Virol, 1998. 72(12): p. 9479-90.
- 20 36. Rothmann, T., et al., J Virol, 1998. 72(12): p. 9470-8.
37. Dobner, T., et al., Science, 1996. 272(5267): p. 1470-3.

38. Hall, A.R., et al., *Nat Med*, 1998. 4(9): p. 1068-72.
39. Shi, Q., et al., *Hum Gene Ther*, 1997. 8(4): p. 403-10.
40. Nelson, J.E. & M.A. Kay, *J Virol*, 1997. 71(11): p. 8902-7.
41. Bergelson, J.M., et al., *Science*, 1997. 275(5304): p. 1320-3.
- 5 42. Xia, et al., *Curr Top Microbiol Immunol*, 1995. 199 (1): p. 39-46.
43. Goldsmith, K.T., et al., *Virology*, 1998. 248(2): p. 406-19.
44. Scaria, A., et al., *Gene Ther*, 1995. 2(4): p. 295-8.
45. Arap, W., et al., *Science*, 1998. 279(5349): p. 377-80.
46. Pasqualini, R., et al., *Nat Biotechnol*, 1997. 15(6): p. 542-6.
- 10 47. Chartier, C., et al., *J Virol*, 1996. 70(7): p. 4805-10.
48. Dion, L.D., et al., *Cancer Gene Ther*, 1996. 3(4): p. 230-7.
49. Dion, L.D., et al., *Gene Ther*, 1996. 3(11): p. 1021-5.
50. Spergel, et al., *Proc Natl Acad Sci USA*, 1991. 88(15): p. 6472-6.
51. Takenawa, et al., *J Natl Cancer Inst*, 1991. 83(22): p. 1668-72.
- 15 52. Eustace, D., et al., *Gynecol Oncol*, 1993. 50(1): p. 15-9.
53. Kong, B., et al., *Gynecol Oncol*, 1996. 63(1): p. 78-84.
54. Murray, E.J., et al., *Mol Cell Biol*, 1991. 11(11): p. 5534-40.
55. Moran, E., *Faseb J*, 1993. 7(10): p. 880-5.
56. Gotoh, A., et al., *J Urol*, 1998. 160(1): p. 220-9.
- 20 57. Bangma, C.H., et al., *Jama*, 1996. 275(11): p. 837-8.
58. Amalfitano et al., *Proc Natl Acad Sci USA*, 1996 93(8): 3352-6.

59. Ferrin, L.J., Genet Eng (N Y), 1995. 17: p. 21-30.
60. Raben, D., et al., Gene Ther, 1996. 3(7): p. 567-80.
61. Garver RI, Jr., et al., Gene Ther, 1994. 1(1): p. 46-50.
62. Yu, D., et al., Cancer Res, 1993. 53(4): p. 891-8.

5 Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Further, these patents and publications are incorporated by reference herein to the same extent as if each individual publication was specifically and individually
10 indicated to be incorporated by reference.

One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with
15 the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed
20 within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. An infectivity-enhanced conditionally-replicative adenovirus, wherein said adenovirus possesses enhanced infectivity
5 towards a specific cell type due to a modification of the HI loop of the fiber knob of a wildtype adenovirus; said modification resulting in enhanced infectivity relative to said wildtype adenovirus, and wherein said infectivity-enhanced conditionally-replicative adenovirus has at least one conditionally regulated early gene, said
10 early gene conditionally regulated such that replication of said infectivity-enhanced conditionally-replicative adenovirus is limited to said specific cell type.

15 2. The infectivity-enhanced conditionally-replicative adenovirus of claim 1, wherein said cell type is a tumor cell.

3. The infectivity-enhanced conditionally-replicative
20 adenovirus of claim 1, wherein said modification to said HI loop results in CAR-independent gene transfer.

4. The infectivity-enhanced conditionally-replicative adenovirus of claim 1, wherein said modification to said HI loop comprises introducing a ligand into said HI loop.

5

5. The infectivity-enhanced conditionally-replicative adenovirus of claim 4, wherein said ligand is selected from the group consisting of physiological ligands, anti-receptor antibodies and cell-specific peptides.

10

6. The infectivity-enhanced conditionally-replicative adenovirus of claim 5, wherein said ligand comprises a tripeptide having the sequence Arg-Gly-Asp (RGD).

15

7. The infectivity-enhanced conditionally-replicative adenovirus of claim 5, wherein said ligand comprises a peptide having the sequence CDCRGDCFC.

20

8. The infectivity-enhanced conditionally-replicative adenovirus of claim 1, wherein said early gene is conditionally regulated by means selected from the group consisting of a tissue-specific promoter operably linked to said early gene and a mutation in said early gene.

9. The infectivity-enhanced conditionally-replicative adenovirus of claim 8, wherein said tissue-specific promoter is from a gene selected from the group consisting of prostate specific antigen, carcinoembryonic antigen, secretory leukoprotease inhibitor and alpha-fetoprotein.

15

10. The infectivity-enhanced conditionally-replicative adenovirus of claim 1, wherein said infectivity-enhanced conditionally-replicative adenovirus carries a therapeutic gene in its genome.

20

11. The infectivity-enhanced conditionally-replicative adenovirus of claim 10, wherein said therapeutic gene is a herpes simplex virus thymidine kinase gene.

5

12. A method of providing gene therapy to an individual in need of such treatment, comprising the steps of:

administering to said individual an effective amount of
10 the infectivity-enhanced conditionally-replicative adenovirus of claim 10.

13. The method of claim 12, wherein said individual
15 has cancer.

14. A method of killing tumor cells in an individual in need of such treatment, comprising the steps of:

20 pretreating said individual with an effective amount of the infectivity-enhanced conditionally-replicative adenovirus of claim 11; and

administering ganciclovir to said individual.

25

15. A method of infectivity-enhanced and conditionally-replicative adenoviral gene therapy in an individual in need of such treatment, comprising the steps of:

administering to said individual a therapeutic dose of an
5 infectivity-enhanced conditionally-replicative adenovirus, wherein
said adenovirus possesses enhanced infectivity towards a specific cell
type due to modification of the HI loop of the fiber knob of a
wildtype adenovirus, wherein said modification results in enhanced
infectivity relative to said wildtype adenovirus, and wherein said
10 infectivity-enhanced conditionally-replicative adenovirus has at least
one conditionally regulated early gene, said early gene conditionally
regulated such that replication of said infectivity-enhanced
conditionally-replicative adenovirus is limited to said specific cell
type.

15

16. The method of claim 15, wherein said
administration is by means selected from the group consisting of
intravenously, intraperitoneally, systemically, orally and
20 intratumorally.

17. The method of claim 15, wherein said individual has cancer.

5

18. The method of claim 15, wherein said cell is a tumor cell.

10

19. The method of claim 15, wherein said modification to said HI loop results in CAR-independent gene transfer.

15

20. The method of claim 15, wherein said modification to said HI loop comprises introducing a ligand into said HI loop.

20

21. The method of claim 20, wherein said ligand is selected from the group consisting of physiological ligands, anti-receptor antibodies and cell-specific peptides.

22. The method of claim 21, wherein said ligand comprises a tripeptide having the sequence Arg-Gly-Asp (RGD).

5 23. The method of claim 21, wherein said ligand comprises a peptide having the sequence CDCRGDCFC.

24. The method of claim 15, wherein said early gene is
10 conditionally regulated by means selected from the group consisting of a tissue-specific promoter operably linked to said early gene and a mutation in said early gene.

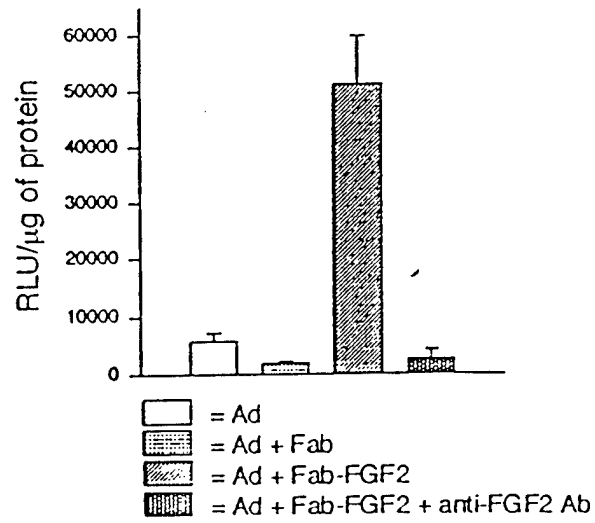
15 25. The method of claim 24, wherein said tissue-specific promoter is from a gene selected from the group consisting of prostate specific antigen, carcinoembryonic antigen, secretory leukoprotease inhibitor and alpha-fetoprotein.

20 26. The method of claim 15, wherein said adenovirus carries in its genome a therapeutic gene.

ABSTRACT OF THE DISCLOSURE

The present invention describes replicative viruses and vectors with improved tumor infectivity and tumor selective
5 replication. The infectivity-enhanced, conditionally-replicative adenoviruses of the present invention are an advanced generation of conditional replicative vectors for cancer gene therapy. Specifically provided is an infectivity-enhanced conditionally-replicative adenovirus, wherein said adenovirus possesses enhanced infectivity
10 towards a specific cell type due to modification of the HI loop of the fiber knob of a wildtype adenovirus, wherein said modification results in enhanced infectivity relative to said wildtype adenovirus, and wherein said infectivity-enhanced conditionally-replicative adenovirus has at least one conditionally regulated early gene, said
15 early gene conditionally regulated such that replication of said infectivity-enhanced conditionally-replicative adenovirus is limited to said specific cell type.

A



B

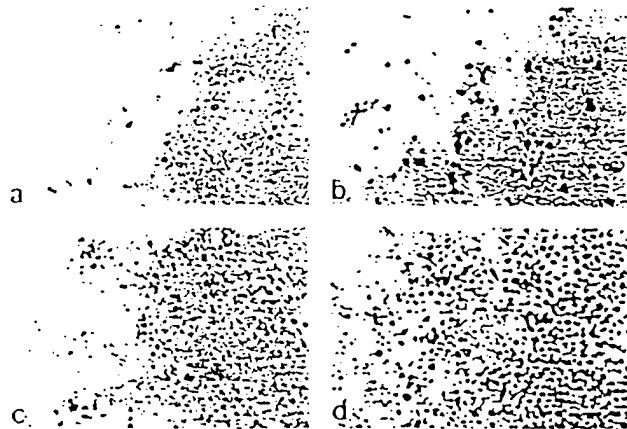


Figure 1

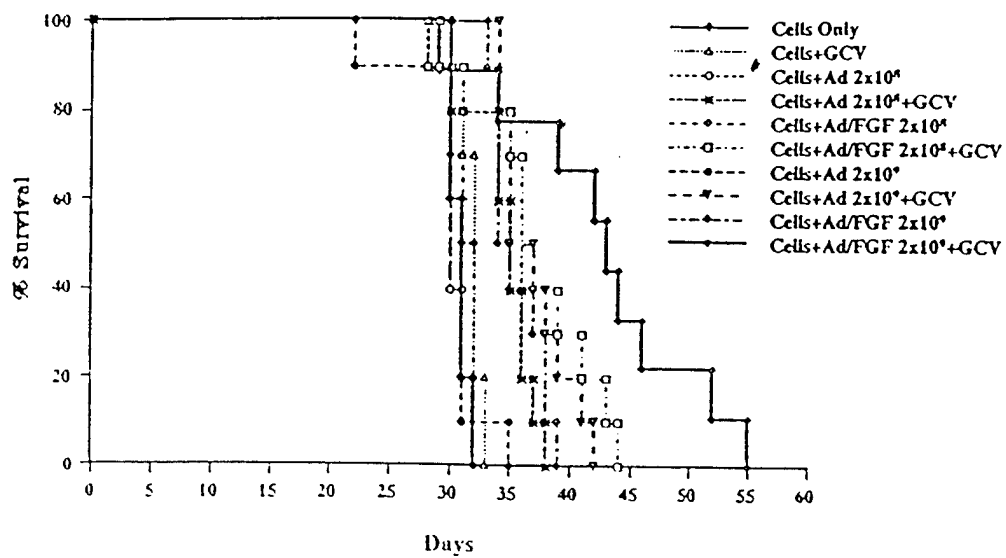


Figure 2

A



B

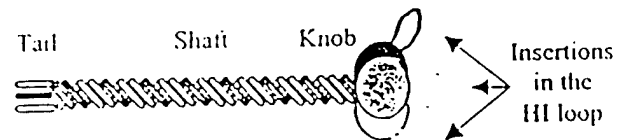
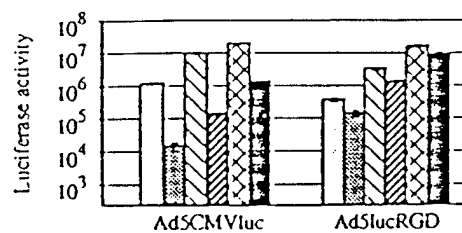
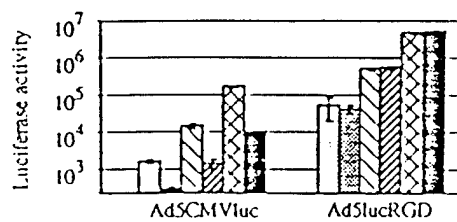


Figure 3

A



B



C

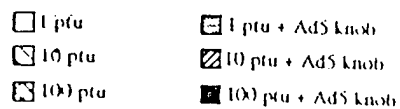
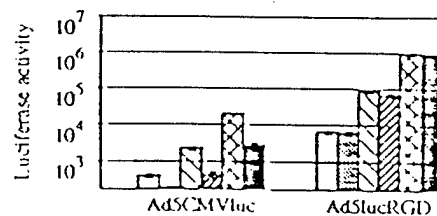


Figure 4

A

B

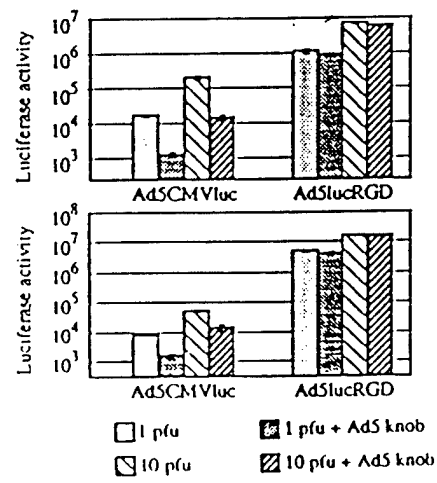
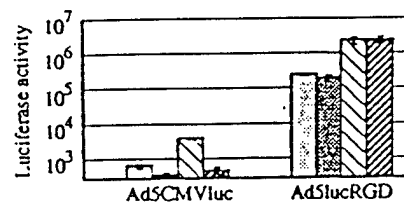
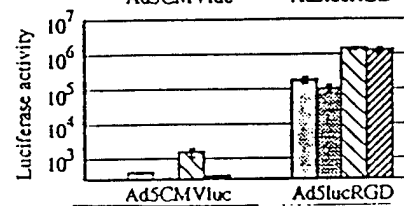


Figure 5

A



B



□ 1 pfu ▣ 1 pfu + Ad5 knob
 ▨ 10 pfu ▩ 10 pfu + Ad5 knob

Figure 6

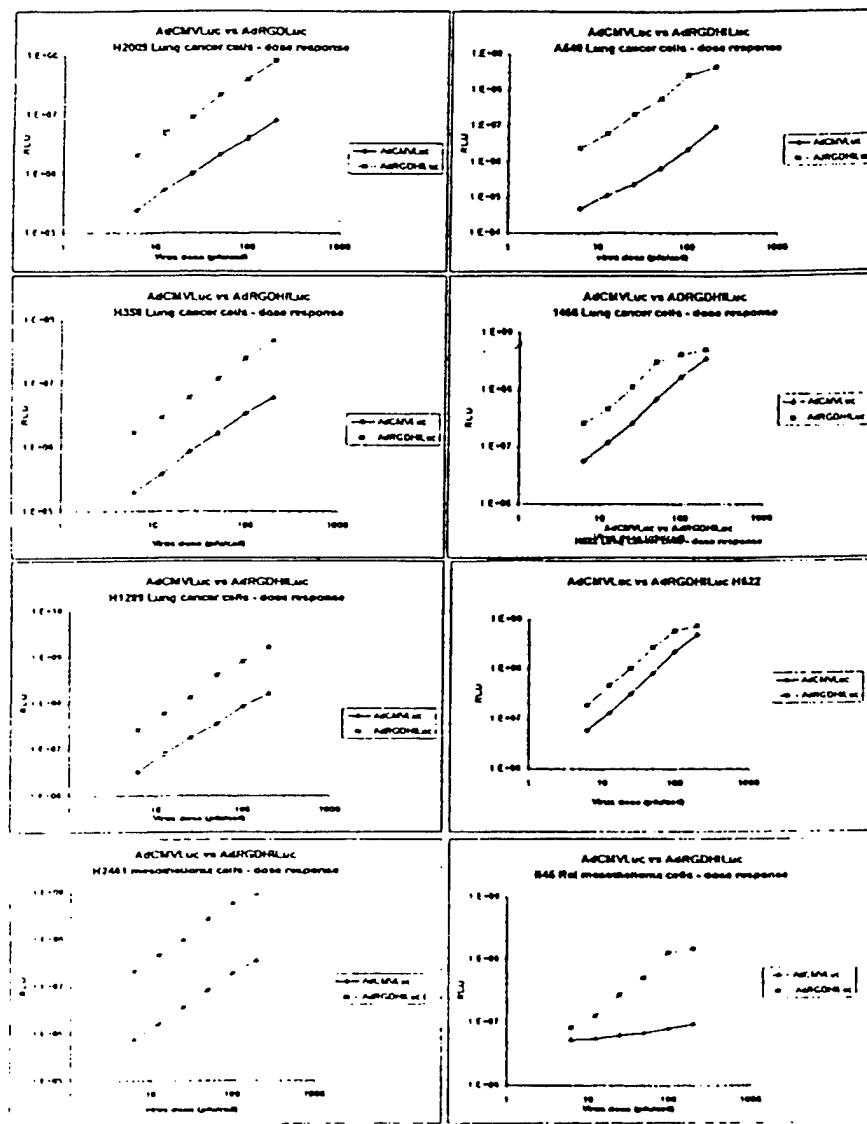


Figure 7

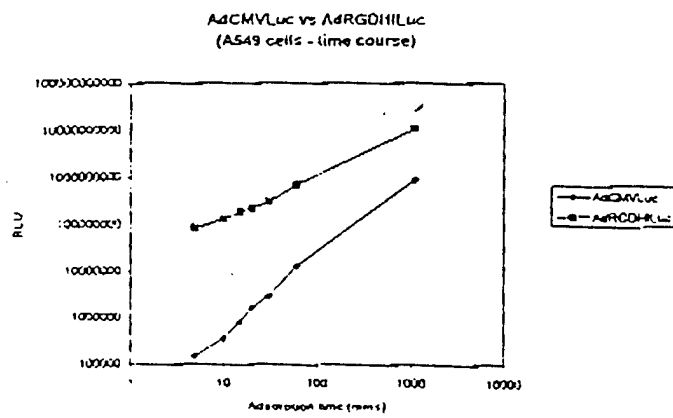


Figure 8

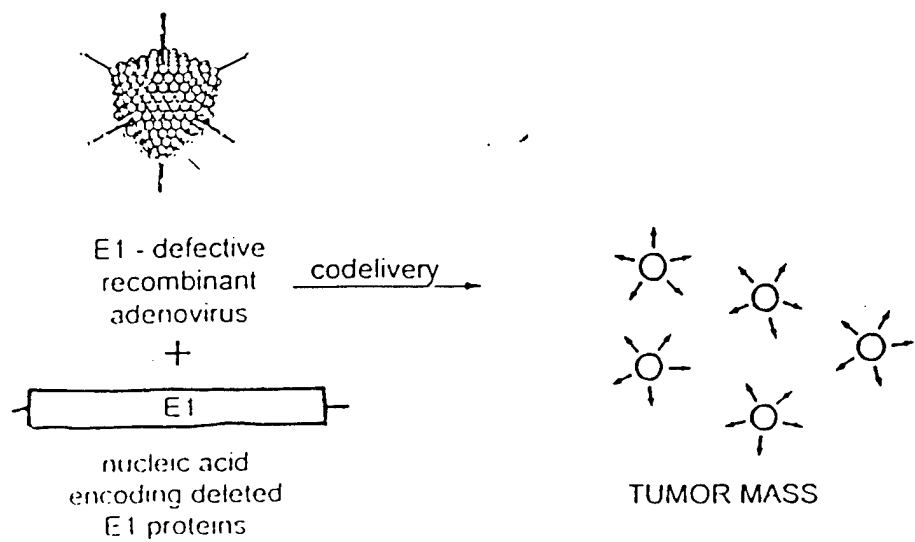


Figure 9

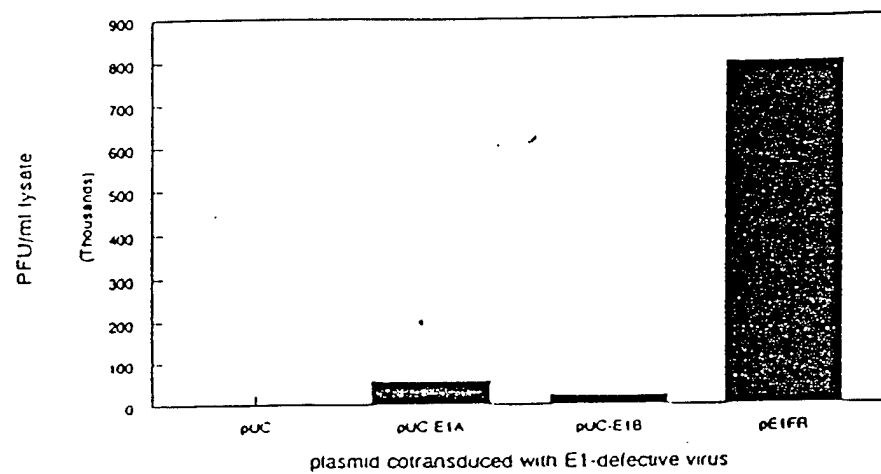


Figure 10

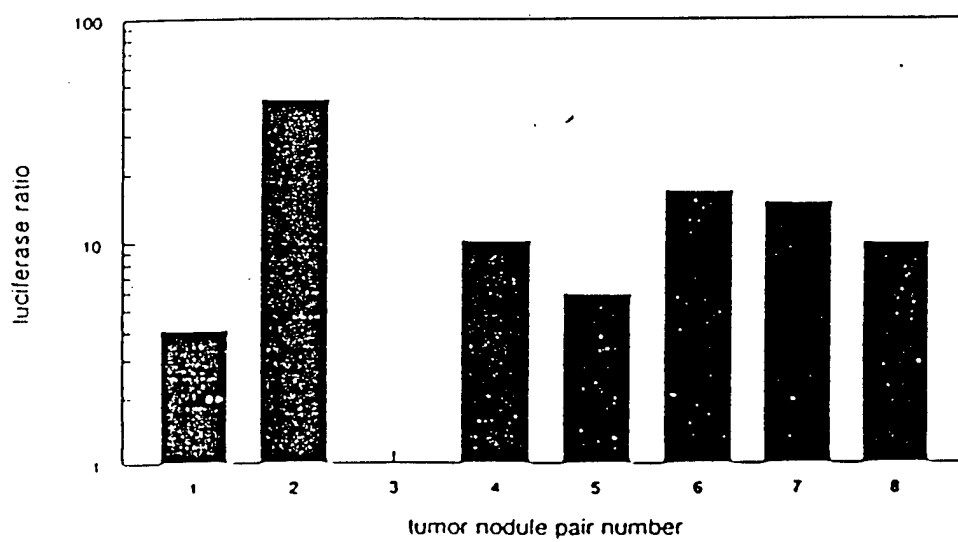


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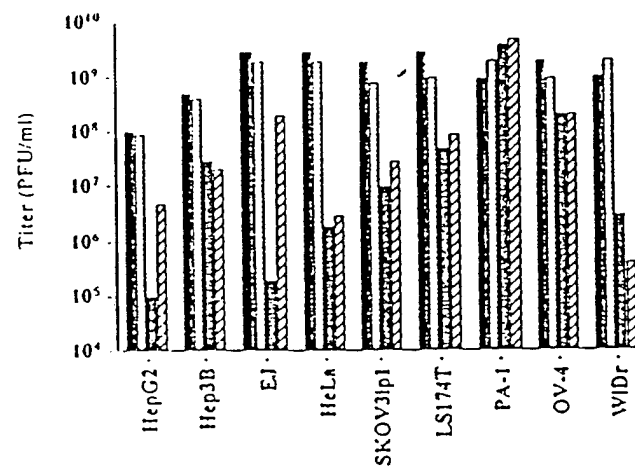
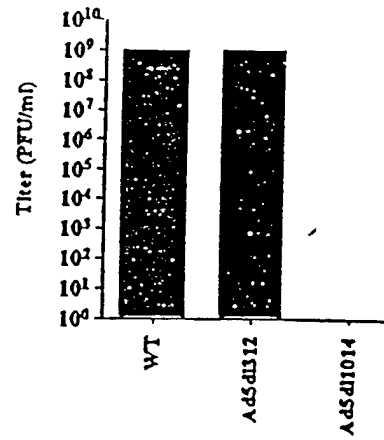


Figure 12

A



B

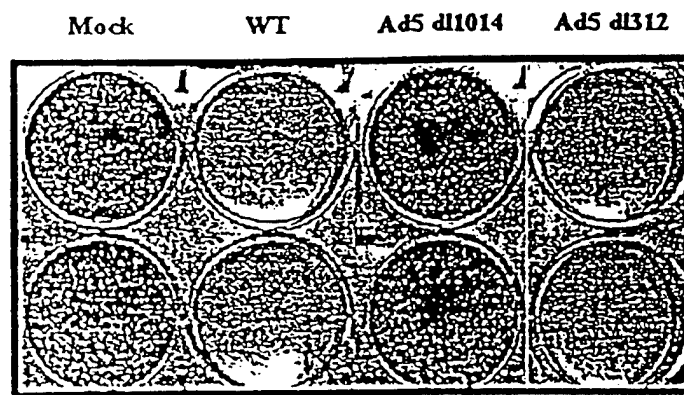


Figure 13

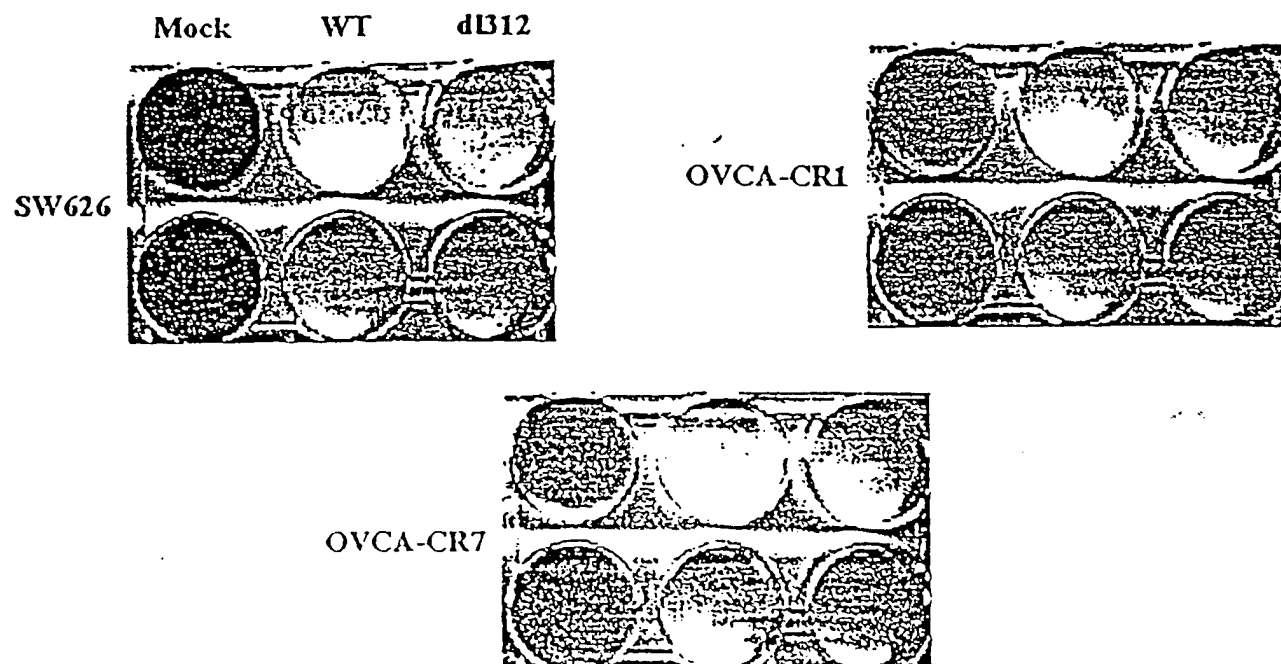


Figure 14

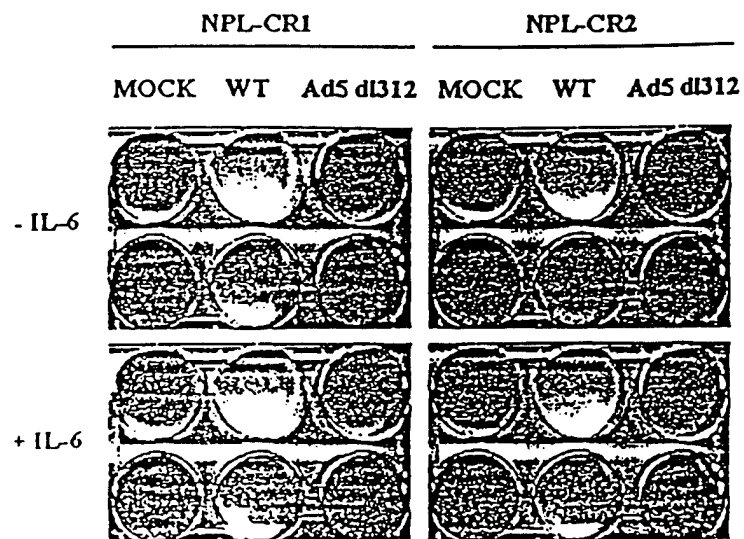


Figure 15